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Investigation of molecular mechanisms and regulatory pathways of pro-angiogenic nanorods†

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Abstract

Angiogenesis, a process involving the growth of new blood vessels from the pre-existing vasculature, plays a crucial role in various pathophysiological conditions. We have previously demonstrated that europium hydroxide [Eu^{III}(OH)₃] nanorods (EHNs) exhibit pro-angiogenic properties through the generation of reactive oxygen species (ROS) and mitogen activated protein kinase (MAPK) activation. Considering the enormous implication of angiogenesis in cardiovascular diseases (CVDs) and cancer, it is essential to understand in-depth molecular mechanisms and signaling pathways in order to develop the most efficient and effective alternative treatment strategy for CVDs. However, the exact underlying mechanism and cascade signaling pathways behind the pro-angiogenic properties exhibited by EHNs still remain unclear. Herein, we report for the first time that the hydrogen peroxide (H₂O₂), a redox signaling molecule, generated by these EHNs activates the endothelial nitric oxide synthase (eNOS) that promotes the nitric oxide (NO) production in a PI3K (phosphoinositide 3-kinase)/Akt dependent manner, eventually triggering angiogenesis. We intensely believe that the investigation and understanding of the in-depth molecular mechanism and signaling pathways of EHNs induced angiogenesis will help us in developing an effective alternative treatment strategy for cardiovascular related and ischemic diseases where angiogenesis plays an important role.

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Introduction

Angiogenesis, the process of recruiting new blood vessels from the pre-existing vasculature, plays a vital role in embryonic growth and development, and in tissue repair, metastatic tumors, rheumatoid arthritis.¹⁻⁴ Impaired angiogenesis is the root cause observed in coronary and ischemic heart diseases (IHD), diabetes, cancer, rheumatoid arthritis, *etc.*⁵ According to the WHO's report in 2011, the cardiovascular diseases (CVDs) lie on top of the list of causes for global deaths among all other non-communicable diseases. One of the treatment strategies for CVDs may be the administration of natural growth factors like VEGF and bFGF for coronary collateral development.⁶ However, these approaches end up with limitations like fibrosis, thrombosis, metastatic cancers⁷ and also a possible significance in disease progression.⁸ Thus there is a serious need for the development of novel alternate approaches for the treatment of CVDs using angiogenic therapy.

In this context, we have demonstrated the pro-angiogenic activity of EHNs that could be used as an efficient nanomedicine treatment strategy for CVDs.⁹⁻¹² Understanding the toxicity and underlying molecular mechanisms behind the pro-angiogenic properties of EHNs is very essential for translating these nanorods to clinical applications for therapeutic purpose. We have already demonstrated the non-toxic nature of EHNs to *in vitro* and *in vivo* systems.¹² However, the exact underlying mechanism and signaling pathways leading the pro-angiogenic activity of EHNs still remain unclear. Our previous reports suggest that generation of ROS especially H₂O₂ might be the plausible mechanism for EHNs induced angiogenesis.^{9,10} There are many inherent signaling pathways, which mediate the process of angiogenesis. One of the key signaling molecules for angiogenesis is the nitric oxide (NO), a low molecular weight and highly diffusible gaseous molecule monitoring many physiological and pathological processes that enables the endothelial cell proliferation, migration and vasodilation. Its implications are seen in various aspects of cardiovascular and diabetic disease mechanisms.¹³ Nitric oxide synthase (NOS) is the major catalytic enzyme which helps in the conversion of cellular L-arginine to L-citrulline, leaving NO as a byproduct. NOS has its existence in three iso-forms eNOS, iNOS and nNOS among which eNOS (endothelial NOS) is very essential for NO production by endothelial cells thereby helping in the simulation of angiogenesis.^{14,15} Earlier reports demonstrate the trigger of eNOS phosphorylation through hydrogen peroxide by various mechanistic signaling pathways.¹⁶⁻¹⁸ In the present study, we report that the generation of H₂O₂ by the EHNs triggers the PI3K/AMPK (AMP-activated protein kinase)/Akt dependent phosphorylation of eNOS enhancing NO production eventually leading to therapeutic angiogenesis. Furthermore the NO induced by the EHNs exerts the angiogenesis in a cyclic GMP (cyclic 3',5'-guanosine monophosphate) mediated fashion. Knowing the in-depth mechanisms underlying the nanorods induced angiogenesis would help towards establishing the development of a novel treatment strategy for CVD related diseases using a nanomedicine approach.

Experimental section

Materials and methods

Materials—Europium nitrate hydrate [Eu(NO₃)₂·H₂O], aqueous ammonium hydroxide [aq. NH₄OH, 28–30%], MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), wortmannin, compound C, *N*^ω-nitro-L-arginine methyl ester hydrochloride (L-NAME), ECM gel and 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) were purchased from Sigma-Aldrich, USA and used without further purification. The fluorescent dyes DAF-FM (4-amino-5-methylamino-2',7'-difluorescein) and DAF-2DA (4,5-diaminofluorescein diacetate) were purchased from Molecular Probes. MnTBAP [Mn(III) tetrakis(4-benzoic acid) porphyrin chloride] and catalase were procured from Calbiochem. Spermine NONOate (SP), 1*H*-[1,2,4] oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ), 8-bromo-guanosine-3',5'-cyclic monophosphate (8-bromo-cGMP) and 2,3,9,10,11,12-hexahydro-10*R*-methoxy-2,9-dimethyl-1-oxo-9*S*,12*R*-epoxy-1*H*-diindolo[1,2,3-*fg*:3',2',1'-*kl*] pyrrolo[3,4-*i*] [1,6] benzodiazocine-10 carboxylic acid, and methyl ester (KT 5823) were purchased from Santa Cruz Biotech. eNOS (49G3) Rabbit mAb and phospho eNOS Ser1177 antibodies were purchased from Cell Signaling Technology whereas anti-Akt/PKB and anti-phospho-Akt (tyr450) antibodies were obtained from Millipore. Goat anti-rabbit and anti-mouse IgG-ALP and FITC conjugate secondary antibodies were purchased from Genei.

Cell culture reagents—The human umbilical vein endothelial cells (HUVECs) and the corresponding endothelial basal media (EBM) supplemented with the endothelial growth media (EGM) kit were procured from Lonza. The fetal bovine serum (FBS), Dulbecco's Modified Eagle's Medium (DMEM), penicillin and streptomycin sulfate were purchased from Sigma-Aldrich. The EA.hy 926 cells were cultured in DMEM complete media. The ECV-304 (eNOS-GFP transfected) cells were a kind gift from Dr V. Shah, Chair, Gastroenterology and Hepatology Department, Mayo Clinic, Rochester, MN, USA.¹⁴

Synthesis and characterization methods

Synthesis and characterization of EHNs using an advanced microwave oven

—EHNs were synthesized as described previously¹⁰ through the interaction of aqueous Eu^{III}(NO₃)₂ and aq. NH₄OH solutions in an advanced microwave oven (SINEOMAS II). In a typical synthesis, 1 mL of aqueous NH₄OH was added to 39 mL of a 0.05 M aqueous solution of Eu^{III}(NO₃)₂ [molar ratio, [OH/Eu] = 4] in a 100 mL round bottomed flask. The reaction mixture was then irradiated at 600 W for 60 min in a microwave oven. The resulting white precipitate was collected through centrifugation at 6000 rpm, washed several times with Millipore water followed by ethanol and Millipore water again. The resulting EHNs solution was then dried overnight in a hot air oven. The as-synthesized nanorods were characterized by various physico-chemical techniques such as XRD, TEM, FTIR, *etc.*

Characterization—Several physico-chemical techniques were employed for the characterization of as-synthesized EHNs including X-ray diffraction (XRD) spectroscopy, transmission electron microscopy (TEM), scanning electron microscopy (SEM), Fourier transform infrared spectroscopy (FTIR), *etc.*

X-ray diffraction (XRD): The phase purity and structural information of the as-synthesized EHNs were determined by XRD analysis using a Bruker AXS D8 Advance Powder X-ray diffractometer (using Cu K α λ = 1.5406 Å radiation).

Transmission electron microscopy (TEM): The shape and morphology of nanorods were examined on a FEI Tecnai F12 (Philips Electron Optics, Holland) instrument operated at 100 kV. Selected area electron diffraction (SAED) patterns were also taken using this instrument.

Dynamic light scattering (DLS): The average particle size distribution and zeta potential (surface charge) of EHNs have been analysed by DLS which are recorded using a Zetasizer Ver. 6.20, Malvern Instruments Ltd.

Fourier transformed infrared spectroscopy (FTIR): FTIR spectroscopic analysis is essential for the identification of functional groups that form a chemical compound on the basis of their bond vibrational energies. The FTIR spectrum is called as “Finger print” of a molecule as the possibility of two compounds having the same FTIR spectra is negligible. The FTIR spectra of the EHNs sample were recorded using a Thermo Nicolet Nexus 670 spectrometer in the diffuse reflectance mode at a resolution of 4 cm⁻¹ in KBr pellets.

Biological methods

Cell culture—The HUVECs were cultured in complete EBM media supplemented with 5% FBS and antibiotics. All the experiments in HUVECs were performed in starved EBM media containing 0.2% FBS to synchronize the effect of treatments in all experiments. The EA.hy 926 and ECV-304 cells were grown in DMEM supplemented with 10% FBS. All the cells were maintained in a 37 °C humidified incubator with 5% CO₂.

MTT assay—Briefly, HUVECs and EA.hy 926 cells were seeded in 96 well tissue culture plates at a density of 10×10^3 cells per well. The cells were incubated with EHNs at varied concentrations (1–20 $\mu\text{g mL}^{-1}$) for 24 h. After incubation, the media in each well were replaced with MTT (0.5 mg mL⁻¹) solution and the plates were kept at 37 °C for 4 h. The MTT solution in each well was then replaced with DMSO: MeOH (1 : 1; v/v) solution to dissolve the insoluble formazan crystals and the absorbance of the samples was measured at 570 nm using a BioTek multimode plate reader.¹⁹

Estimation of nitrite concentration using Griess assay—The Griess assay is a spectrophotometric method based on a diazotization reaction for the detection of nitrite (NO₂⁻) formed as a result of oxidation of NO.²⁰ The EA.hy 926 endothelial cells were seeded in a 24 well plate and grown to confluence. The cells were incubated with EHNs (5 and 10 $\mu\text{g mL}^{-1}$) at different time points (0–12 h). The supernatant media of individual treatments were taken up and mixed with a freshly prepared Griess reagent (1 : 1, v/v) in a 96 well plate and incubated in the dark at room temperature for 20 min. The absorbance of the samples was measured at 540 nm using a BioRad 680 Microplate Reader.²¹ The exact concentration of nitrite produced in EA.hy 926 cells in response to EHNs was calibrated from the nitrite standard curve.

DAF-FM fluorimetry—The HUVECs were seeded in a 24 well plate and grown to confluence. The cells were serum starved for 6 h before incubation with treatments. The HUVECs were then incubated with EHNs ($5 \mu\text{g mL}^{-1}$), catalase (1200 units per mL) and MnTBAP (10 μM) for 4 h. Subsequently the media were replaced with 5 μM DAF-FM and incubated in the dark for 30 min at room temperature. The DAF-FM solution from each of the treatments was transferred to a 96 well microtitre plate and the fluorescence was measured using a fluorescence spectrophotometer^{22,23} with λ_{ex} : 495 nm and λ_{em} : 515 nm.

DAF-2DA fluorescence imaging—DAF-2DA, a non-fluorescent dye having good cell permeability, can measure the NO levels in living cells. The diacetate moieties present in it get hydrolyzed by the intracellular esterases and on reacting with NO it gets converted to a fluorescent triazole derivative whose fluorescence can be measured at an excitation at 488 nm with emission at 515 nm. The EA.hy 926 cells were seeded upon coverslips in a 24 well plate at about 40–50% confluence. The cells were incubated with EHNs ($5 \mu\text{g mL}^{-1}$), catalase (1200 units per mL), MnTBAP (10 μM) and L-NAME (200 μM) for 6 h, followed by incubation with 5 μM DAF-2DA for 30 min. Subsequently, the cells were washed with PBS and the coverslips were mounted onto an Olympus microscope for DAF fluorescence imaging.²⁴

Immunofluorescence—The endothelial cells (HUVECs and ECV-304) were seeded onto coverslips in a 24 well plate at around 50–60% confluence. The cells were incubated with EHNs ($5 \mu\text{g mL}^{-1}$), catalase (1200 units per mL) and MnTBAP (10 μM) for 4 h followed by processing for immunofluorescence. The cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X followed by blocking the nonspecific sites using 5% bovine serum albumin. Then the cells were incubated with phospho-eNOS Ser 1177 primary antibody overnight at 4 °C and the next day with goat anti-rabbit IgG FITC conjugate secondary antibody for 2 h in the dark at room temperature. The cells were washed several times to remove the unbound antibody solutions and incubated with DAPI for 10 min for nuclear staining. Finally, the fluorescence images of the stained cells were captured by using an Olympus fluorescence microscope.^{25,26}

RNA isolation and reverse transcription (RT)—RNA was extracted from HUVECs using TRIzol reagent (Sigma) according to manufacturer's instructions with minor modifications. Briefly, 5×10^6 endothelial cells were pelleted and incubated with 1 ml of TRIzol reagent and incubated for 5 minutes. Cells were then mixed with 0.2 ml of chloroform for 5 minutes at room temperature and centrifuged at 11 500 rpm for 15 minutes at 4 °C. The upper aqueous phase of the sample was then collected in RNase-free Eppendorf tubes and mixed with 0.5 ml of isopropanol for 10 minutes. Samples were then centrifuged at 11 500 rpm for 15 minutes at 4 °C. The supernatant was then aspirated, and the pellet was resuspended in 75% ethanol in DEPC- H_2O by vortexing. Samples were then air-dried, and RNA quantity was measured via nanodrop at 260 nm. RNA integrity was checked by 1% agarose gel electrophoresis. DNase treatment was further carried out to remove DNA contamination from isolated RNA. cDNA was prepared from isolated RNA using 1 μl of RevertAid RT, 5 μg RNA was incubated with 1 μl dNTP mix (10 mM), 4 μl 5 $\times$ Reaction Buffer (250 mM Tris-HCl (pH 8.3), 250 mM KCl, 20 mM MgCl_2 , 50 mM DTT) and 10

picomoles of oligo dT and RNase free water. Total 10 µl reaction mixture was initially denatured at 72 °C for 3 min followed by sudden cooling for 10 min in ice after extension was carried out at 42 °C for 60–90 min.

Real time polymerase chain reaction (PCR) (Q-PCR)—We designed all primers by using primer 3 software (<http://frodo.wi.mit.edu/>) and synthesized them commercially (Eurofins, India). Detailed sequences of all primers used in this study have been summarized in Table S1 of the ESI.[†] Ten nanograms of cDNA were analysed on a Step One Plus (Applied Biosystem) using Absolute SYBR Green ROX PCR Master Mix (Takara). Fold-change analysis was based on normalization by GAPDH.

Western blotting (immunoblot)—HUVECs were treated with EHNs in a dose-dependent (5–20 µg mL⁻¹) manner (for 24 h). Both HUVECs and EA.hy 926 cells were treated in a time-dependent (15–120 min) manner using EHNs at a dose of 5 µg mL⁻¹. The total protein from each treatment was obtained by lysing the cells in lysis buffer containing a RIPA buffer and a protease inhibitor cocktail (PIC) mixture. The cellular components were centrifuged at 10 000 rpm in a 4 °C micro-centrifuge for 15 min and the supernatant was collected. The protein concentration of the supernatant was measured by the Bradford assay and equal concentrations of proteins (50 µg) were separated on a 10% SDS-PAGE and transferred to a nitrocellulose membrane (Thermo Scientific). The membrane was then blocked in TBS-T (Tris: 10 mM, pH 7.5; 150 mM NaCl, 0.05% Tween 20) containing 5% non-fat milk for 2 h to prevent non-specific antibody binding. Subsequently, the membrane was washed thoroughly with TBS-T and incubated with primary antibodies such as anti-phospho-eNOS (Ser 1177), anti-eNOS, anti-phospho-Akt (Tyr 450), and anti-Akt overnight at 4 °C. The membrane was again washed with TBS-T and incubated with goat anti-rabbit IgG ALP or goat anti-mouse IgG ALP for 1 h at room temperature. Finally, the immunoblot was developed by the colorimetric method using BCIP/NBT solution.

Chick embryo angiogenesis (CEA) assay—The fertile eggs of brown leghorn were procured from government poultry and incubated in an egg incubator. On the fourth day of incubation, the shell was peeled on the top and a small window was created. Sterile filter paper discs were soaked in the wortmannin (10 nM), Compound C (40 µM) and L-NAME (3 mM) solutions and placed around the blood vessels in the window area. The treated eggs were incubated in horizontal egg incubators (Southern) at 37 °C and 60–70% RH. The images were captured at 0 h and 4 h post treatments using a Sony camera attached to a stereo-microscope. The new blood vessel growth in response to the treatments was quantified using Angio-Quant software.²⁷

Scratch wound healing assay—The ECV-304 cells were seeded in a 24 well plate and grown to confluence. The cells were scratched by using a sterile autoclaved tip in the centre of each well and washed carefully with DPBS. The cells were then incubated with various treatments (10 µM ODQ, 10 µM 8-Br-cGMP, 1 µM KT 5823, 5 µg mL⁻¹ EHNs and 10 ng mL⁻¹ VEGF) up to 4 h. Imaging at 0–4 h using an Olympus microscope monitored the

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migration of the cells at the site of the wound and quantification of the closure of the wound area was done using the ImageJ software.²⁷

Tube formation assay—Briefly, ECV-304 cells were seeded in a 24 well plate at a density of 2×10^4 cells per well. The cells were incubated with EHNs ($5 \mu\text{g mL}^{-1}$), L-NAME ($100 \mu\text{M}$), SNP ($500 \mu\text{M}$) and L-NAME ($100 \mu\text{M}$) along with EHNs ($5 \mu\text{g mL}^{-1}$) for 24 h. Following this, the cells were trypsinized, collected and seeded in a 96 well plate pre-coated with ECM gel overnight. The formation of tube-like structures was observed by using a digital Nikon microscope.

Statistical analysis—The statistical analysis of all the data was performed using the unpaired Student's *t*-test and “One way ANOVA” with Dunnett's post-test performed using Graph-Pad Prism Version 6.04, San Diego, California, USA (*significantly different from control at $P < 0.05$; **significantly different from control at $P < 0.001$; ***significantly different from control at $P < 0.0001$).

Results and discussion

Synthesis and physico-chemical characterization of EHNs

EHNs have been synthesized according to our published literature¹⁰ through the interaction of aqueous $\text{Eu}^{\text{III}}(\text{NO}_3)_2$ and aq. NH_4OH solutions in an advanced microwave oven (SINEOMAS II) (Fig. S1[†]). The synthesized nanorods (EHNs) are characterized by various physicochemical techniques such as X-ray diffraction (XRD), Transmission electron microscopy (TEM), Fourier transformed infrared spectroscopy (FTIR), *etc.* The XRD pattern reflects the crystalline nature of the EHNs (Fig. 1a). All the diffraction peaks can be indexed to a pure hexagonal phase.¹⁰ The TEM image of EHNs (Fig. 1b) clearly indicates their rod shaped structure. The particle size distribution of EHNs has been calculated using ImageJ software that shows that average length and width of nanorods are around 150–200 nm and 40–50 nm, respectively (Fig. S2a–b[†]). The DLS analysis further supports that the average particle size distribution of EHNs is around 200 nm (Fig. S2c[†]). The zeta potential of the EHNs as measured by DLS is $+30.4 \pm 5.6$ mV indicating a positive surface charge (Fig. S2d[†]). The FTIR spectra of these nanorods show two characteristic peaks at 3610 cm^{-1} and 705 cm^{-1} which may be assigned due to the $-\text{OH}$ stretching frequency and deformation of $\text{Eu}-\text{OH}$, respectively (Fig. S3[†]).²⁸ In order to investigate the pro-angiogenic activity of EHNs towards an *in vitro* system, we have incubated several endothelial cells (HUVECs, EA.hy 926 cells and ECV-304 cells) in the presence of these nanorods and performed several *in vitro* assays such as cell viability (MTT) assay, Griess assay, DAF-FM fluorimetry, DAF-2DA fluorescence imaging, immunofluorescence, western blotting (Immunoblot), and scratch assay. *In vivo* angiogenesis of EHNs has been performed using the chick embryo angiogenesis (CEA) assay.

EHNs enhance endothelial cell proliferation

The cell viability assay is a preliminary assay to analyze the growth and viability of cells in response to treatments. The cell viability assay of endothelial cells (HUVECs and EA.hy 926) treated with EHNs using the MTT reagent demonstrates an increase of endothelial cell

proliferation in a dose dependent manner up to $10 \mu\text{g mL}^{-1}$, suggesting the enhanced cell growth and viability due to EHNs treatment (Fig. 1c and d).

EHNs increase intracellular nitrite and nitric oxide levels

It is established that nitrite is a biological metabolite of nitric oxide (NO) oxidation and its physiological store.²⁹ Therefore, measurement of nitrite levels will reflect the cellular NO production. The Griess assay has been carried out for the estimation of time-dependent nitrite production in EA.hy 926 cells triggered by EHNs ($5\text{--}10 \mu\text{g mL}^{-1}$) that reveals the maximum nitrite level at 4 h post EHNs treatment in comparison with other time points (Fig. 2a and b).

Moreover, it has been found that nitrite levels become significantly higher in cells treated with $5 \mu\text{g mL}^{-1}$ EHNs compared with $10 \mu\text{g mL}^{-1}$ dose due to which all further experiments have been carried out using a lower optimum dose ($5 \mu\text{g mL}^{-1}$) of nanorods. Our previous reports demonstrated the role of ROS especially H_2O_2 in EHNs mediated angiogenesis when the HUVECs are incubated with EHNs. Also, we have observed that incubation of HUVECs with EHNs in the presence of MnTBAP (superoxide dismutase mimetic) enhances the formation of H_2O_2 as well as angiogenesis.¹⁰

Based on our previous results and published literature that support the trigger of eNOS phosphorylation by using hydrogen peroxide by various mechanistic signaling pathways,^{16–18} we hypothesize that generation of H_2O_2 by EHNs mediates through PI3K/AMPK/Akt dependent phosphorylation of eNOS and enhances NO production eventually leading to therapeutic angiogenesis (Scheme 1). Hence, incubation of HUVECs using EHNs in the presence of MnTBAP should increase the production of H_2O_2 and activate eNOS leading to the enhancement of NO production, which would become lowered by catalase treatment (Scheme 1). To establish the direct link between H_2O_2 and NO, the levels of NO production have been estimated in HUVECs and ECV-304 treated with EHNs in the presence of MnTBAP and catalase by employing the DAF-FM fluorimetry assay. Fig. 2c and d reveal an increased NO production in both HUVECs and ECV-304 cells, respectively during incubation with EHNs in the presence of MnTBAP whereas NO generation is decreased in the presence of catalase. Spermine NONOate (SP), a nitric oxide donor, has been used as a positive control experiment. Also, VEGF has been used as another positive control experiment as it is reported that VEGF induces the release of NO from endothelial cells.³⁰ Our results clearly signify the direct relationship between the H_2O_2 production and subsequent NO release in endothelial cells induced by EHNs.

EHNs enhance intracellular NO (DAF/DAR) fluorescence

In order to detect the NO signaling molecule produced in endothelial cells upon treatment with EHNs, fluorescence microscopy has been performed. Fig. 3a–f and S4[†] represent the fluorescence images of EA.hy 926 cells incubated with the following treatments: (a, a1) control, (b, b1) EHNs, (c, c1) EHNs + MnTBAP, (d, d1) Spermine NONOate (SP) ($10 \mu\text{M}$), (e, e1) L-NAME, (f, f1) EHNs + L-NAME, (g, g1) EHNs + catalase and (h, h1) EHNs + MnTBAP + catalase for 4 h followed by incubation with DAF-2DA for 30 min. The images clearly support the preceding results as green fluorescence is increased while the cells are

treated with the combination of EHNs and MnTBAP (Fig. 3c and c1) compared with EHNs alone (Fig. 3b and b1) and untreated control experiments. The feeble green fluorescence of untreated EA.hy 926 cells (Fig. 3a and a1) appears may be due to the endogenous NO. The positive control treatment SP shows the intense green fluorescence that supports the higher NO formation (Fig. 3d and d1). In order to confirm the role of eNOS in NO production, the endothelial cells are incubated with an eNOS inhibitor L-NAME in the absence and presence of EHNs (Fig. 3e, e1 and f, f1). Fig. 3e and e1 show the complete absence of green fluorescence in cells due to the presence of L-NAME. However, the green fluorescence improved after the addition of EHNs (Fig. 3f and f1). We have also performed similar experiments in HUVECs using DAF-2DA that support the formation of NO in the presence of EHNs (Fig. S5[†]). In addition, the red fluorescence of HUVECs incubated with (a) control, (b) TE, (c) L-NAME, (d) EHNs (20 $\mu\text{g mL}^{-1}$) and (e) EHNs + L-NAME for 4 h followed by incubation with DAR for 30 min has been measured by fluorescence microscopy (Fig. S6[†]). These results support nitric oxide imaging using DAF-2DA (Fig. S5[†]). Altogether, these results confirm that nanorods induce the formation of NO that takes place through the generation of H_2O_2 mediated through eNOS.

EHNs promote NO production through eNOS activation

Immunofluorescence has been performed in endothelial cells (HUVECs & ECV-304) treated with (a) control, (b) EHNs, (c) EHNs + MnTBAP (M), (d) EHNs + catalase (C), (e) EHNs + MnTBAP + catalase and (f) VEGF in order to examine the phosphorylation status of eNOS^{25,26} induced by the EHNs treatment and the data are presented in Fig. 4a–g (for HUVECs) and Fig. S7[†] (ECV-304). Incubation of HUVECs with the combination of EHNs and MnTBAP shows more green fluorescence (Fig. 4c) compared with the control untreated cells (Fig. 4a) and cells treated with EHNs alone (Fig. 4b). However, fluorescence intensity decreases in HUVECs treated with EHNs in the presence of catalase (Fig. 4d). Again, a slight increase in intensity occurs upon the addition of MnTBAP (Fig. 4e). The corresponding quantification of fluorescence intensities (Fig. 4g) demonstrates an increased phosphorylation of eNOS (Ser 1177 residue) in the presence of SOD mimetic MnTBAP along with nanorods compared with nanorods alone and a significant decrease of eNOS activation in the presence of the H_2O_2 quencher (catalase), which confirms that the H_2O_2 formed by these nanorods plays a direct role in inducing the eNOS phosphorylation. Similarly, immunofluorescence experiments of ECV-304 cells further support the role of H_2O_2 in eNOS phosphorylation (Fig. S7[†]).

Increased eNOS gene expression induced by EHNs

The gene expression of various NOSs has been found to be important for the physiological angiogenesis. eNOS is very essential for the production of nitric oxide and to promote angiogenesis.³¹ To evaluate the effect of EHNs on eNOS along with iNOS mRNA expression, real time-PCR analysis has been performed. The results represented in Fig. 5 demonstrate an increased gene expression of eNOS in HUVECs incubated with EHNs compared with VEGF and untreated controls. The iNOS expression levels diminished in the presence of EHNs treatments. Moreover, iNOS is reported to be expressed as a direct consequence of inflammation.³² The above results strengthen our proof of concept that nitric

oxide production induced by the EHNs is predominantly through eNOS expression and is iNOS independent.

Akt dependent eNOS phosphorylation triggered by EHNs

In order to investigate the phosphorylation status of Akt and eNOS proteins in endothelial cells (HUVECs and EA.hy 926) upon treatment with EHNs, western blot analysis has been carried out and the results are shown in Fig. 6(a–e). Fig. 6a shows an increased Akt phosphorylation in $5 \mu\text{g mL}^{-1}$ and $10 \mu\text{g mL}^{-1}$ treatment doses compared with a higher dose ($20 \mu\text{g mL}^{-1}$) suggesting the increased efficacy at a lower dose. The corresponding densitometric analysis is shown in Fig. S8.[†] Furthermore treatment of endothelial cells (HUVECs & EA.hy 926) with $5 \mu\text{g mL}^{-1}$ of these nanorods in a time-dependent manner (15–120 min) exhibits the maximum phosphorylation of Akt (Tyr 450) and eNOS (Ser 1177) at 30 min post treatment, as shown in Fig. 6(b–e), respectively. The corresponding densitometric analysis is shown in Fig. S9(a, b) and S10(a, b).[†] These results suggest an Akt dependent eNOS phosphorylation in HUVECs induced by these nanorods, which might be one of the plausible mechanisms behind their pro-angiogenic properties, supporting the reported literature.^{16,33}

EHNs trigger NO production through AMPK/PI3K facilitated eNOS activation

The *in vitro* experiments (Fig. 2–6) suggested the role of EHNs in the generation of H_2O_2 that ultimately induces the NO formation through Akt dependent eNOS phosphorylation eventually promoting angiogenesis. In order to understand the molecular mechanism underlying the EHNs mediated angiogenesis in an *in vivo* model, we have performed the CEA assay, a standard *in vivo* assay to study the formation of new blood vessel growth using different parameters.²⁷ The egg yolk has been treated with (a, a1) control, (b, b1) EHNs, (c, c1) wortmannin, (d, d1) wortmannin + EHNs, (e, e1) Compound C, (f, f1) Compound C + EHNs, (g, g1) L-NAME and (h, h1) L-NAME + EHNs, and results are shown in Fig. 7(a–h). In order to understand the role of eNOS, AMPK and PI3K in EHNs mediated *in vivo* angiogenesis, egg yolks are incubated with EHNs in the presence and absence of L-NAME, Compound C (AMPK inhibitor) and wortmannin (PI3K inhibitor). The egg yolks incubated with wortmannin (Fig. 7c and c1), Compound C (Fig. 7e and e1) and L-NAME (Fig. 7g and g1) show lower blood vessel formation compared with those treated with EHNs alone (Fig. 7b and b1). However, blood vessel formation recovered after the addition of EHNs (Fig. 7d, d1, f, f1 and h, h1). Our results suggest that EHNs cause AMPK activation, which in turn activates Akt and eNOS for generating NO mediated angiogenesis. These results corroborate with the reported literature.³⁴ Altogether the results suggest that the angiogenesis is induced by these nanorods through the PI3K/AMPK/eNOS signaling pathway. The various angiogenesis parameters of the blood vessels like length, size and junctions have been quantified as shown in Fig. 7i–k respectively which together show a decrease in the length, size and junctions of blood vessels for L-NAME, Compound C and wortmannin treatments followed by recovery of these essential parameters after the addition of EHNs.

EHNs induced NO acts downstream through the cGMP mediated pathway

NO shows its physiological effects by activating various downstream signaling messengers. It binds with the inactive guanyl cyclase enzyme and forms a NO–guanyl cyclase active complex.³⁵ This complex helps in the conversion of Guanosine-5'-triphosphate (GTP) to cGMP and thus elevates the cGMP levels, thereby initiating the downstream cGMP signaling and its subsequent biological effects.^{36,37}

Cell migration is one of the hallmarks for the angiogenesis along with proliferation and tube formation.²⁷ In the present study, the scratch assay has been employed to investigate the role of cGMP in the NO induced angiogenesis. The ECV-304 cells have been incubated after making a line of scratch, with various treatments like ODQ (inhibitor of cGMP), 8-bromo-cGMP (activator of cGMP) and KT 5823 (Protein Kinase G inhibitor) to check the migration of cells in the wound area of ECV-304 cells up to 4 h (Fig. 8). Pre-incubation with both ODQ and KT 5823 along with EHNs inhibits the wound closure of ECV-304 cells, whereas 8-bromo-cGMP maintains the wound closure in ECV-304 cells. The inhibition of wound closure by ODQ and KT 5823 clearly confirms the formation of NO induced by the nanorods that directs angiogenesis downstream through the cGMP mediated pathway.

Tube formation induced by EHNs

Another vital pre-requisite for pro-angiogenesis is the tube formation of endothelial cells stimulated by growth factors.³⁸ Therefore, the tube formation assay of ECV-304 cells incubated with EHNs has been performed in the presence and absence of L-NAME (Fig. S11[†]). The ECV-304 cells treated with EHNs show an increased tubular structure formation compared with the control untreated ECV-304 cells and cells incubated with L-NAME alone and a combination of L-NAME and EHNs where L-NAME has been used as the inhibitor of NO formation. However, tube formation of ECV-304 cells incubated with the combination of L-NAME and EHNs is higher than that of L-NAME alone due to the presence of EHNs. The results suggest that EHNs enhance the tube formation through increased NO production.

Overall discussion

Nanoparticles have been extensively used for various advancements in the field of biomedical nanotechnology research. The impact of the nanomaterials on the health care system has boosted dramatically due to the typical drawbacks and pitfalls of the existing therapies. Recently, EHNs have been well reported for their pro-angiogenic properties where reactive oxygen species (ROS) especially hydrogen peroxide are shown as the key signaling molecule behind the nanorods induced angiogenesis. The present study aimed to investigate the in depth downstream angiogenesis signaling exhibited by EHNs. The MTT assay using nanorods shows a good linear dose-dependent increase in cell proliferation. The nitric oxide signaling which is a major signaling pathway responsible for physiological angiogenesis is hypothesized to be involved in EHNs mediated angiogenesis. The results of the Griess assay show a maximum amount of nitrite levels at 4 h post treatment with EHNs in endothelial cells (HUVECs and ECV-304). Hence, all the further experiments have been carried out using this time point of treatment. The DAF-FM fluorimetry assay reveals that NO levels are high in endothelial cells treated with EHNs compared with the untreated control. A further

NO increase in the presence of MnTBAP confirms that H₂O₂ induced by the EHNs actually stimulates NO production. To investigate the role of marker proteins like Akt and eNOS involved in EHNs induced NO synthesis, immunofluorescence and western blot analyses are carried out. The results demonstrate an enhanced phosphorylation of Akt dependent eNOS in response to EHNs treatment, which establishes that nanorods stimulate NO synthesis through the activation of eNOS. In order to validate the complete signaling pathway, pharmacological inhibitors for PI3K, AMPK and eNOS have been used along with EHNs and examined for blood vessel formation using CEA, which suggests the vital role of PI3K/AMPK/Akt in eNOS activation. Further investigation of downstream NO signaling by the scratch assay using inhibitors for cGMP, PKG demonstrates the importance of cyclic GMP in EHNs induced angiogenesis mediated by NO. Considering the enormous implication of angiogenesis in our healthcare system, our nanomedicine approach could be useful for the development of a novel therapy for cardiovascular and ischemic diseases in the near future. Previous reports by our group already demonstrated the *in vivo* non-toxic behavior of EHNs¹² and *in vivo* angiogenesis in chick embryo and zebra fish models.^{9,10} Based on the previous reports and present in-depth molecular mechanism study, we have already initiated the application of therapeutic angiogenesis by nanorods in a rat model for the treatment of limb ischemia and brain ischemia. However, the animal study needs rigorous investigation and currently it is beyond the scope of the present work. In the present report, we focused on understanding in-depth molecular mechanisms and signaling pathways in order to develop towards the most efficient and effective alternative treatment strategy for cardiovascular and ischemic diseases.

Conclusion

We report the synthesis of EHNs using an advanced microwave irradiation method. The nanorods exhibit excellent pro-angiogenic properties to various endothelial cells (HUVECs, EA.hy 926 and ECV-304 cells) and *in vivo* systems, as is evident by various *in vitro* as well as *in vivo* assays. The elevation of nitrite and NO levels in endothelial cells incubated with EHNs suggests the EHNs mediated angiogenesis through the NO signaling pathway. Our results established the direct correlation between H₂O₂ and NO through eNOS phosphorylation, mediated by EHNs. The results of fluorescence imaging, immunofluorescence, western blot analysis, CEA assay, scratch assay and tube formation assay altogether confirm that the EHNs mediated angiogenesis occurs through NO formation by phosphorylating Akt and eNOS proteins. Inhibiting PI3K, AMPK and eNOS by pharmacological inhibitors could attenuate angiogenic responses, while EHNs could recover the inhibitory effects of the inhibitors on angiogenesis. Moreover, elevating cGMP levels and activating cGMP mediated signaling pathways have been found to be the downstream angiogenesis signaling pathway mediated by EHNs induced NO. Altogether, EHNs promote angiogenesis by following the ROS-NO-cGMP signaling axis. Finally, we conclude that the in-depth molecular mechanism and understanding of signaling pathways of EHNs induced angiogenesis would assist towards the development of novel treatment strategy for cardiovascular related diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Characterization of EHNs by various physico-chemical techniques. (a) XRD pattern and (b) TEM image of nanorods. Effect of EHNs on the viability of (c) HUVECs and (d) EA.hy 926 cells using the MTT assay.

Fig. 2.

EHNs enhance nitrite levels in endothelial cells. Estimation of nitrite concentration ($\mu\text{g mL}^{-1}$) in EA.hy 926 cells treated with EHNs (a) $5 \mu\text{g mL}^{-1}$ and (b) $10 \mu\text{g mL}^{-1}$, respectively, using the Griess assay. Estimation of direct nitric oxide released by (c) HUVECs and (d) ECV-304 cells incubated with EHNs ($5 \mu\text{g mL}^{-1}$) by using the DAF-FM fluorimetry assay.

Fig. 3. Enhanced NO production by EHNs. Fluorescence imaging of nitric oxide production in EA.hy 926 cells incubated with (a, a1) nothing (control), (b, b1) EHNs ($5 \mu\text{g mL}^{-1}$), (c, c1) EHNs + MnTBAP, (d, d1) Spermine NONOate (SP) $10 \mu\text{M}$, (e, e1) L-NAME and (f, f1) EHNs + L-NAME using the DAF-2DA dye.

Fig. 4. (a–g) EHNs enhance the eNOS phosphorylation. Immunofluorescence of eNOS-phosphorylation at the ser-1177 residue in HUVECs treated with (a) nothing (control), (b) EHNs ($5 \mu\text{g mL}^{-1}$), (c) EHNs + M, (d) EHNs + C, (e) EHNs + M + C and (f) VEGF (here M and C correspond to MnTBAP and catalase, respectively). The quantification of the green fluorescence intensities is represented as a histogram in (g).

Fig. 5.
The NO induced by EHNs is eNOS dependent and iNOS independent. Real-time PCR analysis of eNOS, iNOS, VEGF and VEGFR2 expression in HUVECs incubated with EHNs.

Fig. 6. EHNs cause Akt dependent eNOS activation. Western (immuno) blot analysis of phosphorylation of Akt and eNOS at Tyr 450 and Ser 1177 residues, respectively in HUVECs (a–c) and EA.hy 926 cells (d, e) incubated with EHNs.

Fig. 7.

In vivo angiogenesis assay using the chick embryo model. Chick embryo incubated with (a, a1) nothing (control), (b, b1) EHNs, (c, c1) wortmannin, (d, d1) wortmannin + EHNs, (e, e1) compound C, (f, f1) compound C + EHNs, (g, g1) L-NAME and (h, h1) L-NAME + EHNs respectively and analyzed for angiogenesis. The new blood vessel formation is indicated using black arrows. Quantification of the parameters like blood vessels length (i), size (j) and junctions (k) using AngioQuant software.

Fig. 8.

EHNs exert angiogenesis through the NO-cGMP axis. The scratch (wound healing) assay to analyse the migration of ECV-304 cells incubated with EHNs, ODQ, 8-Br-cGMP and KT 5823 respectively, quantified as a histogram using ImageJ software.

Scheme 1.

Overall graphical representation of the hypothesized molecular mechanisms underlying the EHNs induced angiogenesis mediated through the ROS-NO-cGMP signaling axis.