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Modulation of NO and ROS production by AdiNOS transduced vascular cells through supplementation with L-Arg and BH_4: Implications for gene therapy of restenosis

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Abstract

Objective—Gene therapy with viral vectors encoding for NOS enzymes has been recognized as a potential therapeutic approach for the prevention of restenosis. Optimal activity of iNOS is dependent on the intracellular availability of L-Arg and BH_4 via prevention of NOS decoupling and subsequent ROS formation. Herein, we investigated the effects of separate and combined L-Arg and BH_4 supplementation on the production of NO and ROS in cultured rat arterial smooth muscle and endothelial cells transduced with Ad_{iNOS}, and their impact on the antirestenotic effectiveness of Ad_{iNOS} delivery to balloon-injured rat carotid arteries.

Methods and Results—Supplementation of Ad_{iNOS} transduced endothelial and vascular smooth muscle cells with L-Arg (3.0 mM), BH_4 (10 μM) and especially their combination resulted in a significant increase in NO production as measured by nitrite formation in media. Formation of ROS was dose-dependently increased following transduction with increasing MOIs of Ad_{iNOS}. Exposure of RASMC to Ad_{iNOS} tethered to meshes via a hydrolysable cross-linker, modeling viral delivery from stents, resulted in increased ROS production, which was decreased by supplementation with BH_4 but not L-Arg or L-Arg/BH_4. Enhanced cell death, caused by Ad_{iNOS} transduction, was also preventable with BH_4 supplementation. In the rat carotid model of balloon injury, intraluminal delivery of Ad_{iNOS} in BH_4-, L-Arg-, and especially in BH4 and L-Arg supplemented animals was found to significantly enhance the antirestenotic effects of Ad_{iNOS}-mediated gene therapy.

Conclusions—Fine-tuning of iNOS function by L-Arg and BH_4 supplementation in the transduced vasculature augments the therapeutic potential of gene therapy with iNOS for the prevention of restenosis.

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Keywords

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Introduction

In-stent restenosis (ISR) following stent angioplasty is characterized by vascular smooth muscle cell (VSMC) migration, proliferation and extracellular matrix production [1]. Drug eluting stents (DES) were developed to prevent VSMC growth but lead to increased rates of late stent thrombosis. As an alternative to DES, our group [2-4] and others [5-9] have developed methods of localized gene delivery from stent tethered gene vectors. Gene therapy strategies that increase nitric oxide (NO) bioavailability are attractive targets for the treatment of ISR because of NO's pleiotropic effects of inhibition of platelet adhesion and VSMC proliferation and stimulation of endothelial regrowth. The effectiveness of these therapies has been demonstrated in studies showing reduced neointima formation after angioplasty upon genetic overexpression of different nitric oxide synthase (NOS) isoforms [6, 10-14]. Additionally, cardiovascular disease states are characterized by a loss of NO bioavailability and transgenic expression of NOS enzymes would address this underlying cause.

Inducible nitric oxide synthase (iNOS) generates NO through oxidation of the guanidino nitrogen of L-Arginine (L-Arg). The cofactor, tetrahydrobiopterin (BH_4) promotes iNOS homodimer formation and is necessary for iNOS derived NO generation [15]. In the absence of L-Arg or BH_4, iNOS catalytic activity becomes uncoupled from NO production and superoxide ($\bullet O_2^-$) is formed by the donation of electrons from iNOS directly to molecular oxygen [16]. NOS uncoupling with concurrent BH_4 depletion has been noted in hypercholesterolemic individuals [17]. NO bioavailability can be further reduced by scavenging of NO by $\bullet O_2^-$. The reaction of NO and $\bullet O_2^-$ can result in formation of the potent oxidant peroxynitrite ($ONOO^-$) which has been implicated as a pathogenic factor in cardiovascular disease states [18]. Oxidation of BH_4 results in the formation of 7,8-dihydrobiopterin which does not promote NOS homodimer formation. Such reduction of BH_4 levels can lead to further $\bullet O_2^-$ production by uncoupled iNOS and development of a feed-forward mechanism by which iNOS derived $\bullet O_2^-$ oxidizes BH_4 resulting in further uncoupling of iNOS.

We previously demonstrated the therapeutic effectiveness of stent-based delivery of adenovirus encoding for inducible nitric oxide synthase (Ad_{iNOS}) to reduce levels of restenosis compared to bare metal stents [3]. In this study, delivery of Ad_{iNOS} to the carotid arteries of rats resulted in increased NO production but generation of $\bullet O_2^-$from uncoupled iNOS was not evaluated. The primary target of stent-based gene therapy, VSMC, do not contain significant levels of BH_4 and overexpression of iNOS would be expected to result in increased production of $\bullet O_2^-$. Gene therapy is an attractive option compared with other treatments for ISR because suppression of neointimal growth can be reversed if the inhibiting agent is removed [19] and gene therapy allows for prolonged and even permanent expression of the therapeutic gene. Healing after stent deployment is a long-term process and iNOS derived $\bullet O_2^-$ production may impair vascular repair and result in increased neointima formation at time points beyond the extent of this study. However, overproduction of $\bullet O_2^-$ by uncoupled iNOS can be regulated through oral supplementation with L-Arg and BH_4. Others have previously demonstrated the effectiveness of oral supplementation with BH_4 to enhance NO production and decrease oxidant stress after Ad_{eNOS} gene transfer in the rat hindlimb ischemia model [20]. Supplementation with L-Arg

and BH_4 could aid healing after stent-tethered Ad_{iNOS} delivery by promoting iNOS coupling and production of NO instead of $\bullet O_2^-$ formation from uncoupled iNOS. In this study we investigate regulation of iNOS catalytic activity through exogenous supplementation of Ad_{iNOS}-transduced VSMC and EC with L-Arg and BH_4 and downstream effects of iNOS coupling on NO/ROS balance in arterial cell types and neointimal formation in the rat carotid model of balloon angioplasty.

Materials and Methods

Isolation and culture of rat VSMC and EC

Animal use was approved by the Institutional Animal Care and Use Committee at the Children's Hospital of Philadelphia. VSMC and EC from the aorta of adult male Sprague-Dawley rats were isolated by a modified version of the method described by Kobayashi et al [21]. Isolated VSMC were cultured in DMEM containing 10% FBS, 1% antibiotic-antimycotic, 40 ng/mL PDGF-BB and incubated at 37°C, 5% CO_2, 95% O_2. EC were cultured in EBM-2 media supplemented with 3% rat serum and EGM-2 SingleQuot. Cells were grown to confluence before Ad vectors were added to cells in DMEM containing 10% FBS and 1% antibiotic-antimycotic. Replication-defective type 5 (E1, E3-deleted) Ad_{iNOS}, Ad_{Luc} and Ad_{empty} driving synthesis of mouse iNOS, firefly luciferase or encoding no transgene, respectively, under control of human cytomegalovirus promoter were purchased from the Gene Therapy Core Facility of the University of Iowa (Iowa City, IA) or the Gene Vector Core Facility of the University of Pennsylvania (Philadelphia, PA). BH_4 and NH_4 were purchased from Schircks Laboratories (Jona, Switzerland). L-Arg was purchased from Fisher Scientific. L-012 was obtained from Wako USA (Richmond, VA).

NO measurements

NO production was evaluated by detection of nitrite in cell culture media as described elsewhere [22]. Briefly, cell culture media was removed 24 hrs after addition of supplements and an equal volume of 1% sulfanilamide was added to the media. After 10 min, an equal volume to the original media volume of 0.1% N-1-napthylethylenediamine dihydrochloride (NED) was added and the reaction was allowed to proceed for 10 min. The product of the reaction, azo dye, was measured at 540 nm.

iNOS dimer detection

RASMC and RAEC were grown to confluence and then transduced with 100 MOI of Ad_{iNOS} for 18 hrs. Cells were supplemented with L-Arg (3.0 mM), BH_4 (10 μM) or their combination for 30 min before cells were lysed in 40 mM Bis-Tris propane buffer (pH 7.7), 150 mM NaCl, and 10% glycerol with 25 mM sodium taurocholate containing protease inhibitors on ice for 30 min. Cell lysates were mixed with an equal volume of non-denaturing sample buffer (Santa Cruz Biotechnology) before being resolved by SDS/PAGE on a 5% gel at 4°C, transferred and probed with anti-iNOS (Cayman Chemical).

ROS measurements

For ROS measurements, cell culture media was changed and cells were supplemented with L-Arg and BH_4 18 hrs after Ad transduction. After 30 min incubation with supplements, media was removed and 3.0 μM CM-H_2DCFDA or 5.0 μM DHR-123 in PBS with Ca & Mg was added to the cells. Following 1 hr incubation at 37°C fluorescence was measured at 485/538 nm for CM-H_2DCFDA and 500/538 nm for DHR-123. Direct detection of $\bullet O_2^-$ was conducted with 200 μM L-012 in Krebs-HEPES buffer equilibrated with 95% O_2/5% CO_2. In some experiments, RASMC were pretreated with 100 U/mL of polyethylene glycol-conjugated superoxide dismutase (PEG-SOD) for 1 hr before addition of supplements for 30

min. The cells were then incubated with L-012 for 20 min at 37°C prior to detection of chemiluminescence using a SpectraMax L luminescence microplate reader (Molecular Devices, Sunnyvale, CA). Values were normalized to cell number as quantified by Alamar blue.

Cell viability and proliferation

Cell viability was measured 24 hrs after addition of supplements by ethidium homodimer (EthD-1). Cells were incubated in 5 μM EthD-1 for 30 min at 37°C and then observed under a fluorescence microscope with a rhodamine filter set; fluorescence was also measured at 528 nm excitation and 617 nm emission. Cellular proliferation was measured following overnight Ad_{iNOS} (400 MOI) transduction and subsequent 9 hr incubation with supplements. BrdU was allowed to incorporate for 5 hr before fixation of the cells and detection of BrdU by ELISA (Millipore).

Immobilization of Ad to steel meshes

Ad_{iNOS} was tethered to the surface of 316 stainless steel meshes obtained purchased from Electron Microscopy Sciences (Hatfield, PA) as previously described [3]. Briefly, meshes were successively exposed to aqueous solutions of polyallylamine bisphosphonate with latent thiol groups (PABT), tris (2-carboxyethyl) phosphine (TCEP), polyethyleneimine (PEI) with pyridyldithio groups for amplification of attachment sites [PEI (PDT)] and dithiothreitol (DTT). Next, Ad modified with bifunctional (amine-, thiol-reactive hydrolysable crosslinker (RHC) was covalently attached [3].

Intraluminal delivery of AdiNOS to rat carotid arteries

450 – 500 g male Sprague-Dawley rats were used for this experiment. The common carotid artery was denuded with an angioplasty catheter and Ad_{iNOS} ($5x10^7$ infective viral particles) or an equivalent dose of Ad_{Luc} was delivered intraluminally for 5 min. Some AdiNOS-treated rats were supplemented with 250 mg/kg/day L-Arg and 10 mg/kg/day BH_4 with 0.04% ascorbic acid in their drinking water for two days prior to surgery and 15 days post-surgery. Animals were sacrificed 15 days after vascular gene delivery; carotid arteries were excised and fixed in 4% paraformaldehyde. Arteries were embedded in paraffin, sectioned at 8 microns and stained with an elastic stain kit (Sigma-Aldrich). Morphometric image analysis was conducted with ImageJ (NIH) on five sections from each animal and the results were averaged.

Statistical methods

Results represent the means ± SD unless otherwise noted. Comparison between multiple treatment groups was conducted by ANOVA followed by the Student-Newman-Keuls post-hoc test for pairwise comparisons. Results were considered significant at $p < 0.05$.

Results

Effects of L-Arg and BH_4 supplementation on nitric oxide production in Ad_{iNOS}-transduced arterial cells

Baseline nitrite levels in conditioned medium from primary RASMC were minimal (Fig. 1A). Transduction of RASMC with escalating titers of Ad_{iNOS} (MOIs 100-400) resulted in significant nitrite accumulation in the medium, demonstrating that NO production in this cell type is limited by the presence of NOS synthases rather than by the absolute deficiency of L-Arg or BH_4. However, supplementation of Ad_{iNOS}-transduced RASMC with L-Arg, BH_4 and especially their combination significantly increased NO production by the cultured cells (Fig. 1A) revealing the relative deficiency of L-Arg and BH_4 at the super-physiological

levels of iNOS expression found after Ad_{iNOS} transduction. NO production in both supplemented and non-supplemented RASMC peaked at MOIs 100-200.

A comparable pattern of NO production was found in Ad_{iNOS}-transduced A10 cells, derived from embryonic rat aorta (Fig. 1B). In this cell line, however, L-Arg and BH_4 were absolutely necessary for increased NO production, since very little nitrite accumulation was demonstrated in the non-supplemented Ad_{iNOS}-transduced cells regardless of MOI. In primary endothelial cells cultured from rat aorta (RAEC), BH_4 supplementation was found to be crucial for achieving high levels of NO production after Ad_{iNOS} transduction (Fig. 1C). Interestingly, while L-Arg supplementation alone did not increase NO production in Ad_{iNOS}-transduced RAEC, it greatly amplified the effects of BH_4 supplementation on NO synthesis in this cell type.

The iNOS-specific competitive inhibitor, aminoguanidine, was found to dose-dependently decrease NO formation in Ad_{iNOS}-transduced RASMC (Fig. 1D). The inhibition was more profound (75-95%) for unsupplemented and BH_4-supplemented RASMC compared to LArg and L-Arg/BH_4 supplemented cells (20-70%), reflecting competition between L-Arg and aminoguanidine for the substrate binding site of iNOS. Similar effects of iNOS inhibition on NO production in Ad_{iNOS}-transduced RASMC were demonstrated for the unrelated competitive iNOS inhibitor, 1400W (Fig. S1).

We next determined whether NO production at iNOS expression levels achieved via cellular transduction with stainless steel mesh-immobilized Ad vectors are affected by supplementation with L-Arg and BH_4. Approximately $2x10^9$ Ad_{iNOS} particles (~$1.3x10^7$ infective units) were appended to the polyallylamine bisphosphonate modified steel surface using a hydrolysable cross-linker [3]. Two days after mesh placement onto subconfluent RASMC, nitrite concentrations in the conditioned media of cells treated with Ad_{iNOS} meshes were significantly higher than in the media from cells treated with control Ad_{Luc} meshes (Fig. 1E). BH_4 supplementation resulted in a 3-fold increase of NO production by RASMC transduced with Ad_{iNOS} meshes, whereas no increase of NO synthesis was detected in the BH_4-supplemented cells transduced using control Ad_{Luc} meshes. While L-Arg supplementation alone did not increase NO production by Ad_{iNOS} mesh-transduced RASMC, L-Arg and BH_4 in combination synergistically increased NO production, resulting in an approximately 4.5-fold increase of NO formation in comparison with unsupplemented cells (Fig. 1E). Similar effects were observed in RAEC (Fig. 1F). The independence of NO production from augmentation by L-Arg supplementation in Ad_{iNOS} mesh-transduced cells in contrast to free Ad_{iNOS}-transduced RASMC is most likely due to lower overall levels of iNOS expression.

To identify optimal L-Arg and BH_4 supplementation regimens for supporting NO synthesis in Ad_{iNOS}-transduced RASMC, cells transduced at a MOI of 100 as well as non-transduced RASMC were treated with increasing concentrations of one supplement, while either omitting the other or providing it in excess (Fig. 2A, C). No significant nitrite accumulation was observed in the conditioned media of non-transduced cells regardless of supplementation status. A dose-dependent increase in NO production was observed in both the presence and absence of L-Arg (3.0 mM) in Ad_{iNOS}-transduced RASMC (Fig. 2A). Nitric oxide production was 20-40% higher for L-Arg-supplemented RASMC compared to non-supplemented RASMC across all BH_4 supplementation groups (Fig. 2A). Ad_{iNOS} transduced RASMC devoid of BH_4 supplementation produced small amounts of NO with no apparent dose dependency between L-Arg concentration and NO output (Fig. 2C). In contrast, supplementation of RASMC with BH_4 (10 μM) significantly increased NO production and revealed the dose-dependency of NO production as a function of L-Arg

concentration in the medium (Fig. 2C). Comparable results with increasing concentrations of L-Arg and BH_4 on NO production were observed in Ad_{iNOS}-transduced RAEC (Fig. 2B, D).

To determine if supplementation of Ad_{iNOS} transduced arterial cells with L-Arg and BH_4 promotes iNOS dimer formation a low temperature non-denaturing western blot for iNOS was performed. The presence of the monomeric form of iNOS was detected in all samples that were transduced with Ad_{iNOS} regardless of their L-Arg/BH_4 supplementation state. However, in samples that were supplemented with BH_4 and with L-Arg and BH_4 in combination the presence of the dimeric form of iNOS was detected in both RASMC and RAEC (Fig. 2E, F), suggesting that the increased levels of NO production found after supplementation with BH_4 are due to BH_4 mediated formation of dimeric iNOS.

Effects of L-Arg and BH_4 supplementation on ROS formation in Ad_{iNOS}-transduced cells

Limited availability of L-Arg or BH_4 is a well-recognized cause for NOS decoupling that results in excessive production of damaging oxidant $\bullet O_2^-$ [18]. Therefore, we investigated whether Ad_{iNOS}-mediated gene transfer can lead to the expression of decoupled iNOS and whether this decoupling can be reversed by pharmacological supplementation of L-Arg and BH_4.

ROS levels assessed by DCF fluorescence were found to be dose-dependently increased in RASMC transduced with escalating MOIs of Ad_{iNOS} (Fig. 3A). These results were confirmed using an alternative ROS-sensitive fluorescent probe, DHR-123 (Fig. S2). Increased ROS formation after Ad_{iNOS} transduction of RASMC incubated with the iNOS inhibitor, aminoguanidine, was completely reversed and inhibited by 70% in cells transduced at MOIs of 100 and 400, respectively (Fig. 3B). Exposure of Ad_{iNOS}-transduced RASMC to L-NAME was found to not significantly decrease levels of ROS production (Fig. S3) indicating that eNOS uncoupling does not play an essential role in the observed increases in ROS production after Ad_{iNOS} transduction. Remarkably, RASMC transduction by Ad_{empty}, which possesses an intact ability to infect cells but does not encode for a transgene, did not result in increased ROS production (Fig. 3B), suggesting that increased ROS formation after Ad_{iNOS} transduction is related to the activity of the encoded transgene rather than to the adenoviral infection per se. Moreover, aminoguanidine did not affect ROS levels in Ad_{empty}-transduced cells (Fig. 3B), thus refuting the direct antioxidant effects of aminoguanidine. Supplementation of non-transduced, Ad_{iNOS}- and Ad_{empty}-transduced RASMC with L-Arg and BH_4 revealed the direct antioxidant effects of BH_4 on ROS levels unrelated to the NOS recoupling mechanism since ROS activity was proportionally decreased to the same extent in all groups of differently transduced cells upon BH_4 and L-Arg/BH_4 supplementation (Fig. 3B). L-Arg supplementation alone did not affect ROS levels in non-transduced and Ad_{empty}-transduced cells, but significantly increased ROS production in Ad_{iNOS}-transduced cells co-treated with aminoguanidine in comparison with the respective groups of non-supplemented Ad_{iNOS}-transduced RASMC (Fig. 3B). The latter effect is most probably related to the attenuation of iNOS inhibition by aminoguanidine with the increased intracellular concentration of L-Arg.

Both DCF- and DHR-based assays detects multiple ROS entities. To single out the effects of L-Arg and BH4 supplementation on the prevalence of $\bullet O_2^-$, a direct product of uncoupled NOS, the $\bullet O_2^-$ sensitive probe L-012 was used in conjunction with PEG-SOD to determine the proportion of the signal attributable to $\bullet O_2^-$. For this experiment RASMC were supplemented with tetrahydroneopterin (NH_4) in addition to L-Arg and BH_4 to determine if reduced $\bullet O_2^-$ production following BH_4 supplementation of Ad_{iNOS} transduced RASMC is due to the antioxidant properties of BH_4 or is mediated by direct interaction of iNOS and BH_4. NH_4 has similar antioxidant properties to those of BH_4, but is unable to regulate iNOS catalytic activity through dimer formation. L-012-enhanced chemiluminescence results

demonstrate that while BH_4 and L-Arg/BH_4 supplementation of Ad_{iNOS} transduced RASMC was able to significantly reduce levels of ROS, NH_4 alone or in combination with L-Arg was unable to significantly decrease ROS production (Fig. 3C), suggesting that reduced levels of $\bullet O_2^-$ observed after BH_4 supplementation of Ad_{iNOS} transduced RASMC is the result of increased iNOS dimer formation. A similar experiment was conducted in the presence of PEG-SOD to directly evaluate the effect supplementation has on $\bullet O_2^-$ production. The presence of PEG-SOD resulted in a significant decrease of L-012 chemiluminescence in all groups except those supplemented with BH_4 (Fig. 3D). The inability of PEG-SOD to decrease levels of L-012 chemiluminescence in BH_4 supplemented groups suggests that BH_4 supplementation prevents production of $\bullet O_2^-$ from iNOS through promotion of iNOS homodimer formation and coupling of the enzyme.

In RASMC transduced with Ad_{iNOS} tethered to meshes, ROS-specific fluorescence (DCF) was detected in the cells adjacent to the meshes (Fig. 3E) confirming our hypothesis that the cells brought in immediate contact with surface-immobilized Ad_{iNOS} (such as the cells adjacent to implanted gene eluting stents) experience the highest levels of iNOS over-expression and thus may need pharmacological intervention to prevent decoupling of the enzyme. Indeed, in the experiment that involved L-Arg and BH_4 supplementation to Ad_{iNOS} mesh-transduced RASMC, both spread and intensity of DCF-detected ROS signal were attenuated in the BH_4 and LArg/BH_4-cotreated cells compared to the cells receiving L-Arg-only or no supplementation (Fig. 3F).

Effects of L-Arg and BH_4 supplementation on cell death and proliferation in Ad_{iNOS}-transduced cells

Early apoptotic death after vascular injury associated with revascularization procedures is pivotal for triggering a compensatory proliferative response leading to the formation of neointima [23]. Since iNOS overexpression can cause cell death through increased NO [24] and ROS [25] formation we investigated the effects on cell death of both free and mesh-tethered Ad_{iNOS} and their modulation by L-Arg and BH_4 supplementation in RASMC. The number of dead and dying cells stained by EthD-1, which is excluded from live cells, was significantly increased in RASMC transduced with Ad_{iNOS} (MOI 400) in comparison with similarly Ad_{Luc}-transduced RASMC as assessed by fluorescence microscopy and fluorometry (Fig. 4A, B). Whereas L-Arg supplementation did not affect the number of dead cells, BH_4 and especially combined L-Arg/BH_4 supplementation decreased cell death in Ad_{iNOS}-transduced RASMC (Fig. 4A, B). The protective effects of BH_4 and L-Arg/BH_4 supplementation were also observed in Ad_{iNOS}-transduced RAEC (Fig. 4C). In RASMC transduced using mesh-tethered Ad_{iNOS}, cell death was observed in cells adjacent to the meshes. These cells exhibit the highest transgene expression levels [3] and thus are subjected to increased levels of oxidative stress due to Ad_{iNOS} delivery and transduction. The damaging impact of oxidative stress was prevented by BH_4 and L-Arg/BH_4 supplementation (Fig. 4D, E), while massive cell death resulting in cell monolayer defects (Fig. 4D) was apparent in non-supplemented Ad_{iNOS}-transduced RASMC.

The anti-proliferative effects of NO on VSMC are well documented[26]. To determine the role of L-Arg and BH_4 supplementation on RASMC proliferation after Ad_{iNOS} transduction levels of BrdU incorporation were evaluated. Ad_{iNOS} (400 MOI) transduction resulted in a 57% decrease of cellular proliferation as compared to Ad_{empty} (400 MOI) transduced cells. L-Arg and L-Arg/BH_4 supplementation of Ad_{iNOS} transduced cells resulted in a further 46% and 53% reduction of proliferation respectively as compared to non-supplemented Ad_{iNOS} transduced cells. However, under these experimental conditions, BH_4 supplementation alone did not result in a significant decrease in proliferation as compared to non-supplemented Ad_{iNOS} transduced cells (Fig 4F).

Effects of L-Arg and BH_4 supplementation on restenosis in Ad_{iNOS}-transduced balloon-injured rat carotid arteries

The rat carotid balloon injury model was employed to investigate whether supplementation of L-Arg and BH_4 can aid in the prevention of restenosis after therapeutic delivery of Ad_{iNOS}. Naïve rats or rats pretreated for 2 days with L-Arg, BH_4 or L-Arg/BH_4 through medicated drinking water underwent balloon angioplasty injury followed by local intraluminal delivery of Ad_{iNOS} or Ad_{Luc}. Ad_{Luc} transduction was found to result in the highest levels of restenosis (neointima/media ratio 1.49 ± 0.118; Fig. 5A). The extent of restenosis was found to be slightly lower in Ad_{iNOS} transduced arteries (1.334 ± 0.072; Fig. 5B). Supplementation of Ad_{iNOS} transduced animals with L-Arg (1.075 ± 0.107; Fig. 5C), BH_4 (1.086 ± 0.07; Fig. 5D) and L-Arg/BH_4 (0.957 ± 0.084; Fig. 5E) was found to result in a significant decrease in neointima/media ratio as compared to AdLuc transduced arteries (Fig. 5F). Moreover, combined L-Arg/BH_4 supplementation and of Ad_{iNOS} transduced arteries resulted in a significant decrease in neointima/media ratio as compared to Ad_{iNOS} transduction alone.

Discussion

The antirestenotic effects of therapies aimed at increasing NO bioavailability in angioplastied arteries are well established [10] and are generally attributed to a unique combination of the inhibitory effects exerted by NO on platelet adhesion and SMC proliferation and stimulation of endothelial regrowth. Approaches based on genetic over-expression of key enzymes operational in the NO synthesis pathway (NOS, GTPCH1, DHFR) are interesting alternatives to the conventional low molecular drugs and NO eluting polymers, since permanent modification of vascular tissue may offer lasting protection against restenosis and obviate the need for repeated interventions. Recently, we [2, 3] and others [6, 7] have shown that endovascular stents can provide a convenient platform for therapeutic delivery of viral and non-viral gene vectors driving expression of NOS isoforms.

Previous work from several research groups has firmly established the potential clinical relevance of pharmacological intervention with L-Arg and BH_4 for enhancing NOS functionality as a primary or auxiliary therapy for diseases in which dysregulation of NOS plays a prominent pathogenic role (reviewed in [27]). The rationale for L-Arg and BH_4 supplementation for the optimization of NOS coupling is especially relevant in the setting of NOS gene therapy since highly elevated levels of NOS expression achievable with gene transfer should be matched with super-physiological concentrations of L-Arg and BH_4. To our knowledge no study to-date has systemically investigated the concept of combined L-Arg/BH_4 supplementation in the setting of eNOS or iNOS gene therapy for the treatment of restenosis. Therefore, in the current study we examined the effects of combined supplementation with L-Arg and BH_4 to vascular cells transduced by Ad_{iNOS}.

Evidence of elevated NO synthesis upon combined L-Arg and BH_4 supplementation was found in rats with modeled chronic renal failure [28] and in Plasmodium berghei-infected mice [29]. Although the increased expression of iNOS is presumably the main reason behind elevated NO production in both studies, this was not directly shown. In our work we used well controlled conditions of Ad_{iNOS} transduction and direct cell culture supplementation and were able to demonstrate a synergistic L-Arg and BH_4 dose-dependent (Fig. 2) increase of NO synthesis in transduced RASMC and RAEC supplemented with both compounds. NO output was dose-dependent on Ad_{iNOS} levels in the range of 0- 200 MOI, but plateaued at higher MOIs (Fig. 1A-C). The most plausible explanation for the observed L-Arg/BH_4 synergism is the concept that both the substrate and co-factor are relatively deficient in cells experiencing extremely high iNOS expression due to adenoviral transduction. Alternatively, the enhancement of NO production may be related to the increased affinity of L-Arg to the

substrate-binding site of an entirely functional dimeric form of NOS caused by increased BH_4 concentrations [30]. To this end, combined L-Arg/BH_4 dietary supplementation was shown to be significantly more efficient than individual L-Arg and BH_4 regimens in restoring impaired NO-mediated vasoreactivity of atherosclerotic mouse aortas [31].

Aminoguanidine is a competitive inhibitor of iNOS that competes with L-Arg for the substrate-binding site of the enzyme. In accordance with the mode of action for aminoguanidine, inhibition of NO production was partially reversed in Ad_{iNOS}-transduced RASMC supplemented with L-Arg and combined L-Arg/BH_4 (Fig. 1D) validating iNOS as the main source of NO in the transduced cells.

Decoupling of NOS enzymes as consequence of a stochiometic mismatch between enzyme copy number and accessibility of BH_4 and L-Arg, is a well-known mechanism of reduced NO bioavailability and ensuing endothelial dysfunction [32]. Our results (Fig. 2 E, F and 3 B, C and E) support the hypothesis that iNOS gene transfer has to be accompanied by BH_4 supplementation to prevent decoupling of the enzyme. In our analysis of the effect of L-Arg/BH_4 supplementation on the dimeric state of iNOS (Fig. 2 E and F) we found that while BH_4 promoted iNOS dimer formation there was still a significant proportion of the enzyme that was in the monomeric form. With this large monomeric pool of iNOS it could be expected that BH_4 supplementation would not be able to result in the increased NO production (Fig. 2) and reduced ROS formation (Fig. 3B, C) that we observed. However, it is likely that the majority of this monomeric pool of iNOS is not active as depletion of BH_4 has been demonstrated to result in increased association of monomeric eNOS with caveolin-1 and inactivation of the enzyme [33].

In our study, MOI-dependent increase of ROS formation following RASMC transduction with escalating titers of Ad_{iNOS} (Fig. 3A) does not reflect oxidative stress associated with adenoviral transduction per se, but is primarily related to overexpression of iNOS since 1) no enhanced production of ROS was detected in cells transduced with matching titers of Ad_{empty} (Fig. 3B) and 2) increased ROS production with Ad_{iNOS} transduction was inhibited by aminoguanidine (Fig. 3B). Aminoguanidine inhibition of iNOS was found to result in a less robust decrease of ROS production (66.2 % inhibition) than NO production (97.7 % inhibition) in non-supplemented RASMC transduced with 400 MOI $Ad_{iNOS.}$ This discrepancy is possibly due to a decreased affinity of aminoguanidine for uncoupled iNOS as compared to coupled iNOS due to conformational differences between uncoupled and coupled iNOS. While aminoguanidine has been demonstrated to decrease $\bullet O_2^-$ production from uncoupled iNOS the kinetics of this inhibition have not been studied. Previous studies have demonstrated differing effects of the endogenous methylarginines on NOS function based on the coupling state of the enzyme[34]. As aminoguanidine and the endogenous methylarginines both act through competitive inhibition it is possible that aminoguanidine inhibition of uncoupled iNOS is divergent from inhibition of coupled iNOS. Furthermore, while a fraction of ROS-inhibiting activity of supplemented BH4 derives from its general antioxidant effects (Fig. 3 B), the main mechanism of decreasing $\bullet O_2$- production in Ad_{iNOS}-transduced cells is through the promotion of iNOS coupling, as evidenced by the lack of significant reduction of $\bullet O_2^-$ formation in Ad_{iNOS} transduced RASMC supplemented with NH4 (Fig, 3 C), a substance with similar antioxidant properties, but having no effects on iNOS enzyme function.

Attachment of Ad_{iNOS} vectors to the surface of stainless steel meshes, to model vascular tissue transduction with a stent-based gene delivery system, resulted in the selective formation of ROS in cells underlying or immediately adjacent to the meshes (Fig. 3E). These cells were shown in our previous work utilizing reporter vectors to attain a higher transgene expression levels than the rest of the cells in the culture [3]. Since cellular

processes occurring at the stent/artery interface are pivotal for the control of restenosis and stent thrombosis [35], the prevention of NOS decoupling and ROS formation through combined BH_4 therapy can bear significant consequences for the clinical translation of iNOS gene eluting stents [2-9]. While no previous work has demonstrated modulation of ROS synthesis by BH_4 in the context of iNOS-based gene therapy, the capability of exogenously added BH_4 to mitigate oxidative stress was demonstrated in numerous experimental and clinical settings involving upregulation of iNOS expression [32]. Our current work confirms and further expands these findings by showing that BH4 and combined L-Arg/BH4 supplementation, but not L-Arg treatment alone, significantly decreases total ROS (Fig. 3 B) and specifically $\bullet O_2^-$ production (Fig. 3 C, D).

Early ROS-mediated apoptotic death of medial SMC triggered by angioplasty-induced vascular trauma has been shown to contribute to the signaling pathways leading to proliferation and migration of surviving medial cells and to the formation of neointima [23]. Since iNOS overexpression has been shown to affect cell death via ROS-dependent mechanisms [25] we investigated the effects of L-Arg and BH_4 supplementation on survival of Ad_{iNOS} transduced RASMC. In our study, Ad_{iNOS} transduction resulted in a 40% increase in cell death as compared to Ad_{Luc}-transduced and untransduced RASMC (Fig. 4A, B), which was partially reversed by LArg and BH_4, and especially by the combined L-Arg/BH4 regimen. Likewise, BH_4 and combined L-Arg/BH_4 supplementation reversed death of RASMC locally transduced with mesh-immobilized AdiNOS (Fig. 4D, E). These findings are in accordance with the recently established anti-apoptotic effects of BH_4 precursor, sepiapterin, in fetal pulmonary artery endothelial cells isolated from sheep with pulmonary hypertension [36].

Proliferation of Ad_{iNOS}-transduced RASMC, as assessed by BrdU incorporation in S-phase cells, was found to be decreased by 57% in comparison to Ad_{empty}-transduced RASMC, which is in accordance with previous reports showing anti-proliferative effects of NO on VSMC [26]. Interestingly, this inhibition was further augmented by L-Arg and L-Arg/BH4 supplementation, but not with BH4 supplementation alone. This finding indicates that anti-proliferative effector mechanisms in Ad_{iNOS}-transduced RASMC are independent of heightened superoxide production and ROS-initiated signaling.

Our therapeutic study, comparing neointimal formation in rats locally delivered with Ad_{iNOS} following standard balloon injury, did not demonstrate a significant reduction of restenosis in non-supplemented Ad_{iNOS} treated animals as compared to Ad_{Luc} treated animals (Fig 5 A, B and F). This is in contrast with a prior study [14] that showed the anti-restenotic effectiveness of Ad_{iNOS} transduction of balloon-injured rat carotid arteries. A principal difference between our findings and the published study is a 25-fold higher vector dose, employed in our experiments to model a mismatch between iNOS expression and intracellular levels of L-Arg and BH4. Supplementation of AdiNOS-treated rats with L-Arg (Fig. 5 C), BH4 (Fig. 5 D) and especially L-Arg/BH4 (Fig. 5 E) through medicated water rescued the anti-restenotic effectiveness of local vascular Ad_{iNOS} gene transfer (Fig. 5 F). These results corroborate the requirement of iNOS to be in a coupled enzymatic state for the realization of the full therapeutic potential of NOS-based gene therapy for vascular restenosis.

In conclusion, we demonstrated that NO production, as well as the formation of ROS following Ad_{iNOS}-mediated gene transfer of vascular cells can be pharmacologically regulated in a dose dependent manner by concurrent L-Arg and BH_4 supplementation. Additionally, our supplementation regimens were demonstrated to regulate cell viability and proliferation after Ad_{iNOS} transduction and the *in vivo* therapeutic anti-restenotic effects of iNOS after delivery to arterial tissue. The pharmacological modulation of transgene function

after viral delivery may be a useful concept to help allow for safer and more controllable implementation of vascular gene therapy for clinical use.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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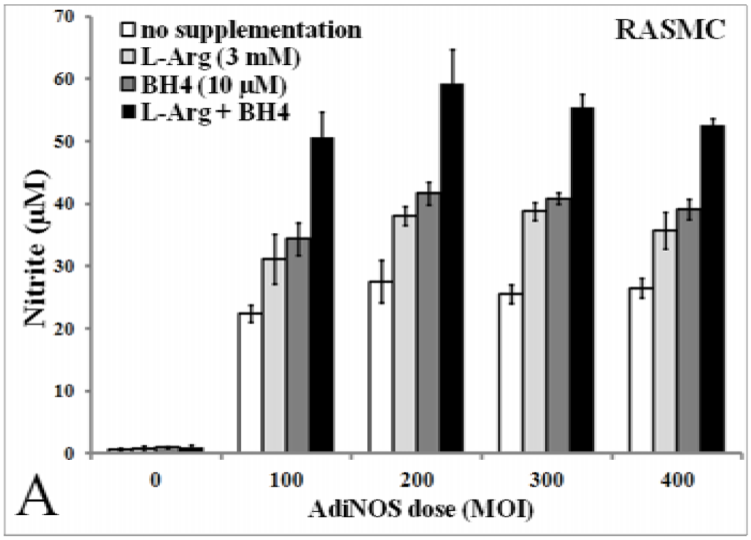
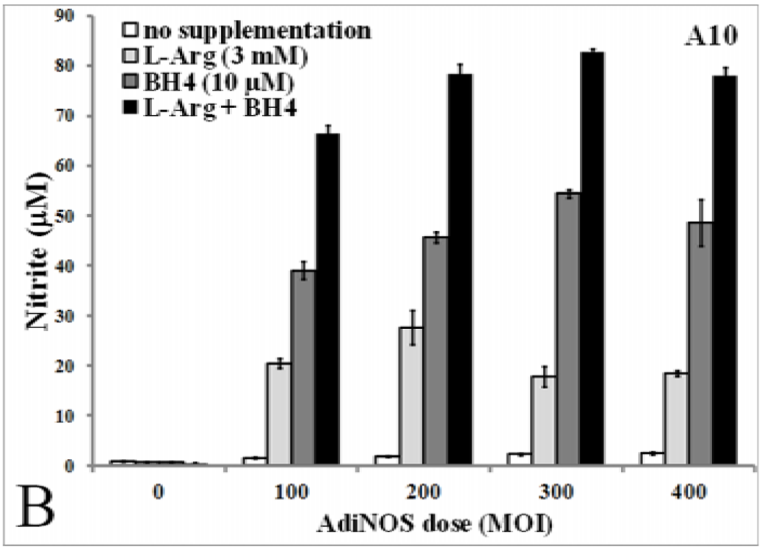
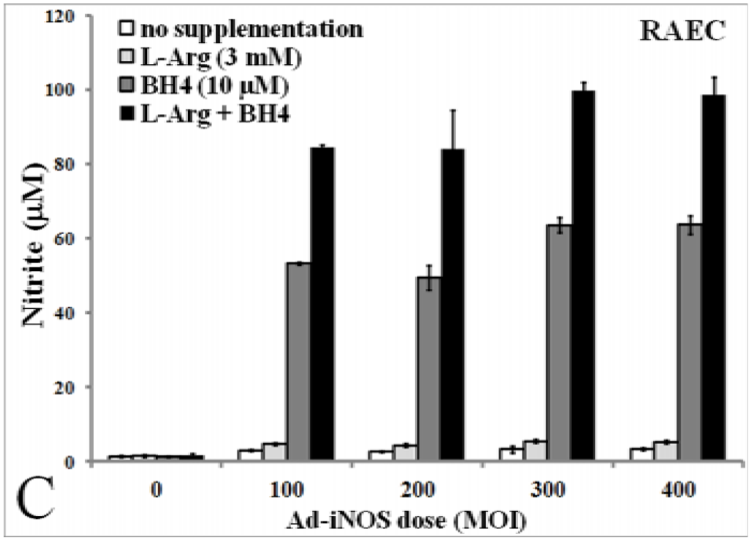
Highlights

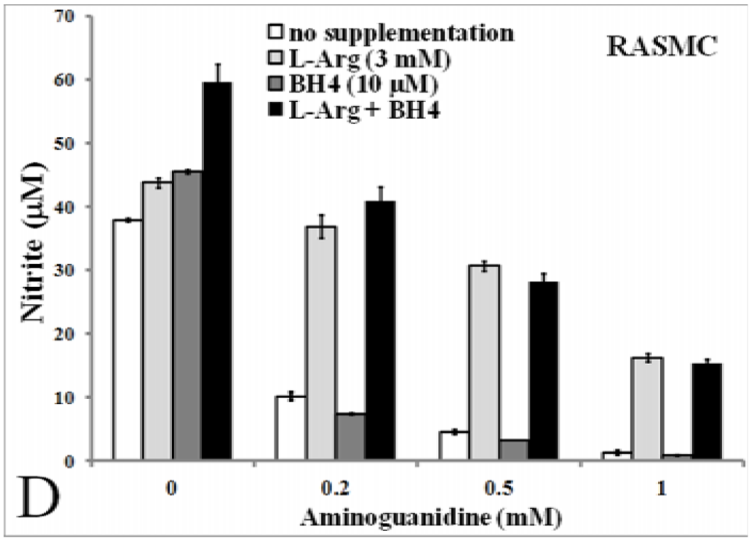
- L-Arg and BH4 promote NO production in Ad_{iNOS} transduced RASMC and RAEC
- BH4 decreases ROS production in Ad_{iNOS} transduced RASMC and RAEC
- BH4 promotes iNOS dimer formation in Ad_{iNOS} transduced RASMC and RAEC
- L-Arg and BH4 reduce levels of restenosis in Ad_{iNOS} transduced rat carotid arteries

- Generation of nitric oxide by rat aortic smooth muscle and endothelial cells transduced with free and surface-immobilized Ad_{iNOS} is synergistically augmented by L-Arg and BH4 supplementation
- ROS formation by Ad_{iNOS}-transduced rat aortic smooth muscle and endothelial cells is decreased by BH4, but is either not affected or increased by L-Arg supplementation
- Increased NO generation and reduced ROS production by Ad_{iNOS} transduced cells is mediated by enhanced enzymatic coupling after BH4 supplementation.
- In rats undergoing carotid balloon denudation injury followed by intraluminal Ad_{iNOS} delivery, intravital BH4 supplementation results in higher *ex vivo* arterial NO production in comparison to non-supplemented controls.
- L-Arg and BH4-mediated normalization of the NO/ROS balance in arteries treated with Ad_{iNOS}-eluting stents may be a clinically applicable combined gene/drug therapy approach for the prevention of in-stent restenosis









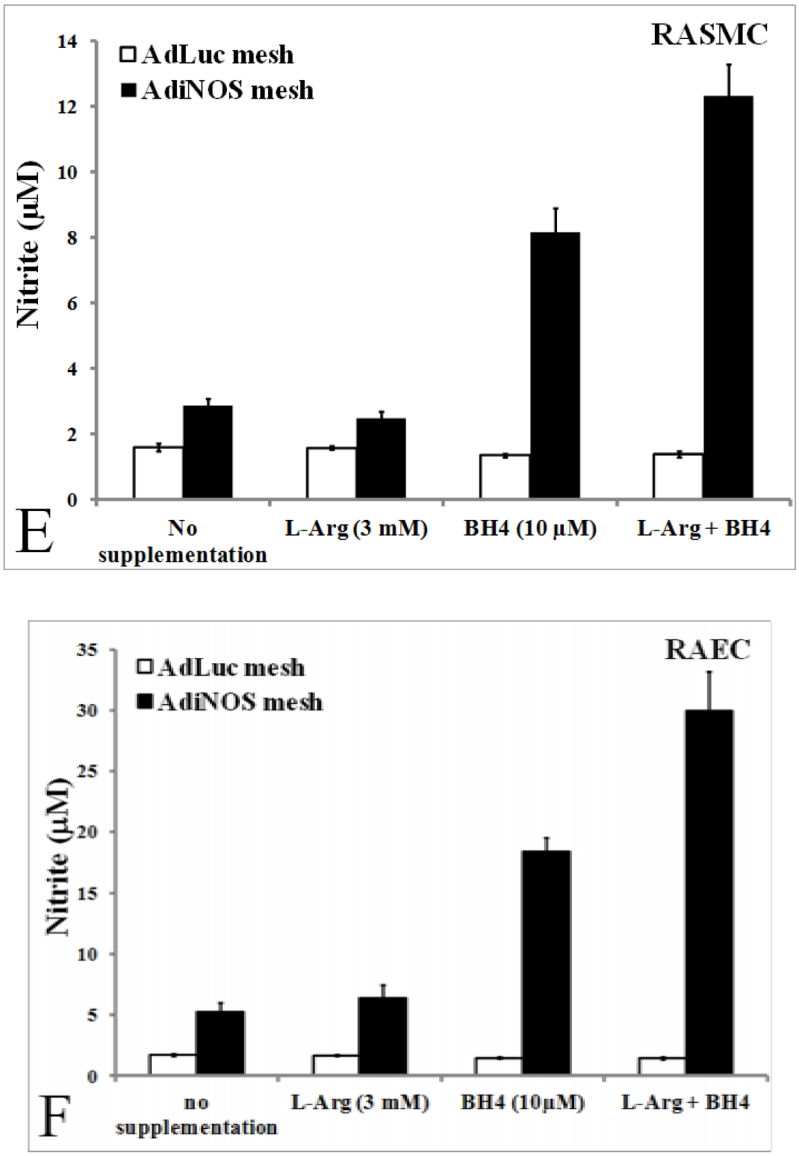
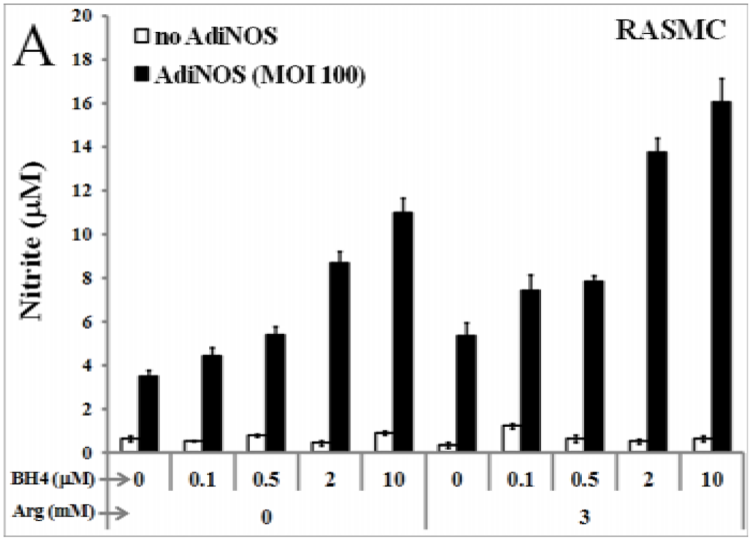
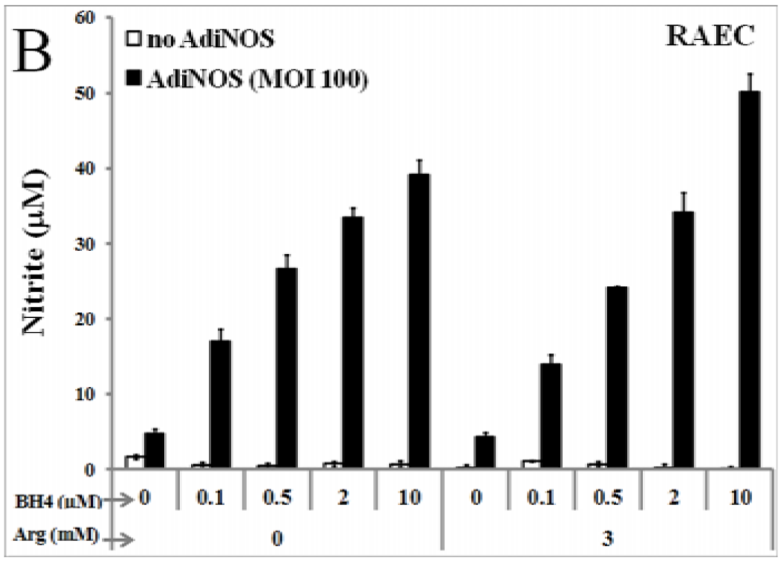


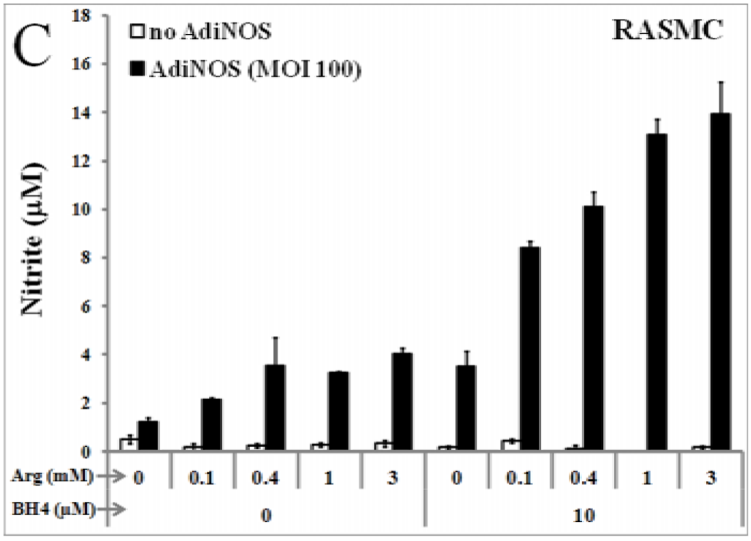
Figure 1. NO production from AdiNOS transduced RASMC and RAEC

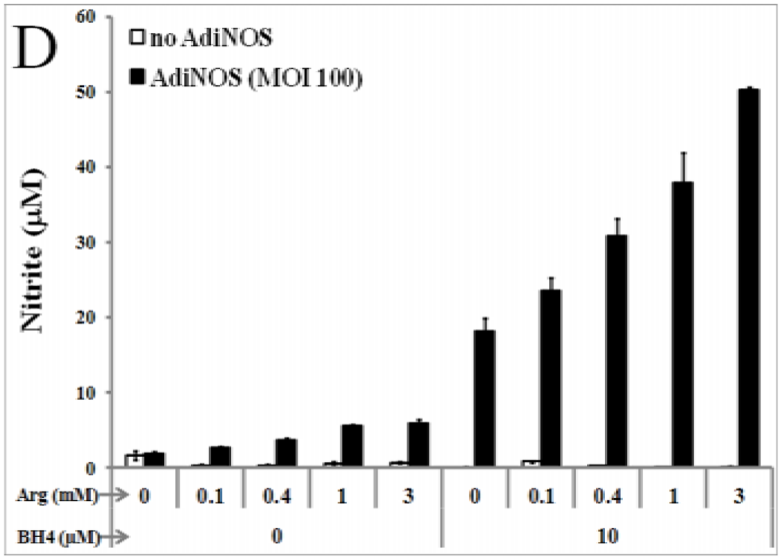
Nitrite levels in conditioned cell culture media were determined by the Griess assay 24 hrs after addition of L-Arg (3.0 mM) and BH_4 (10 μM) and 42 hrs after transduction with increasing MOIs (0 – 400) of Ad_{iNOS} in primary RASMC (A), A10 cells (B) or primary RAEC (C). Primary RASMC transduced with Ad_{iNOS} (100 MOI) were exposed to Aminoguanidine (0 – 1.0 mM) for 1 hr prior to addition of L-Arg/BH_4 (D). Ad_{Luc} or Ad_{iNOS} tethered meshes were placed on arterial cells and nitrite levels in conditioned cell culture media was determined 24 hrs after addition of L-Arg/BH_4 and 42 hrs after mesh placement on RASMC (E) and RAEC (F).





RAEC





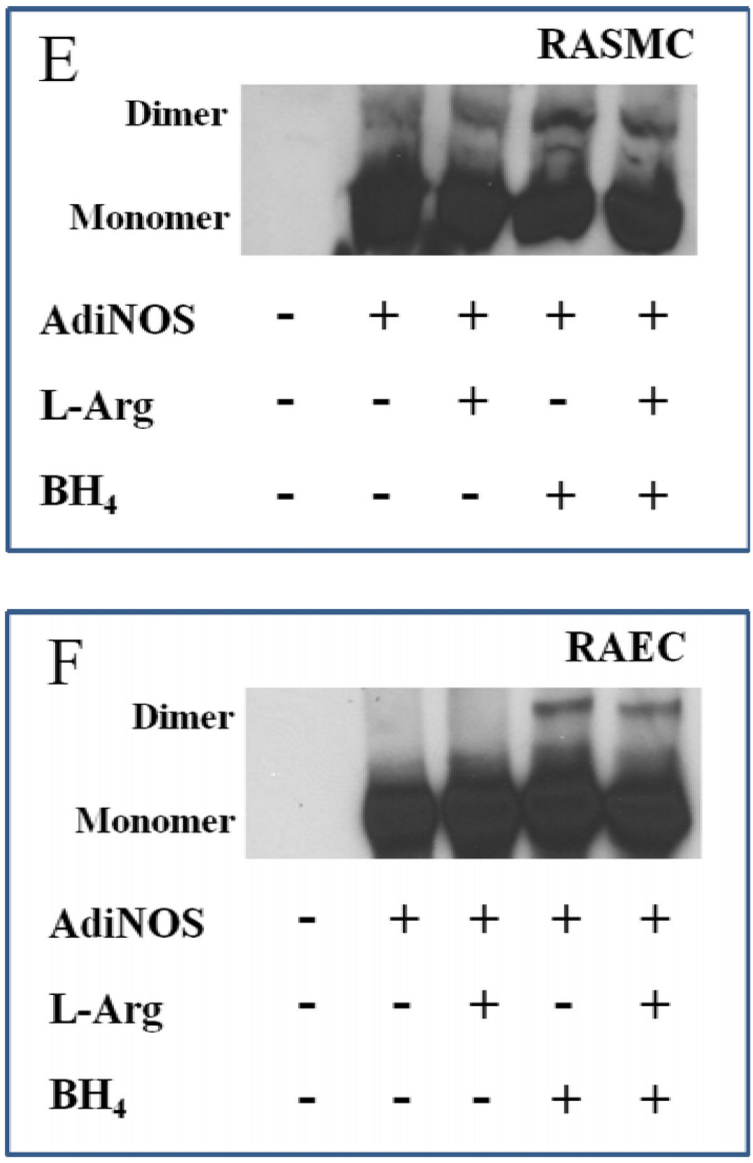
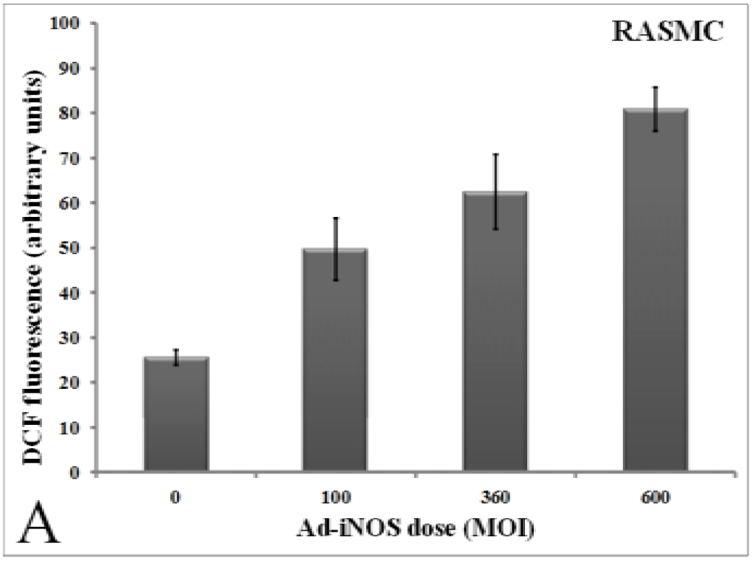
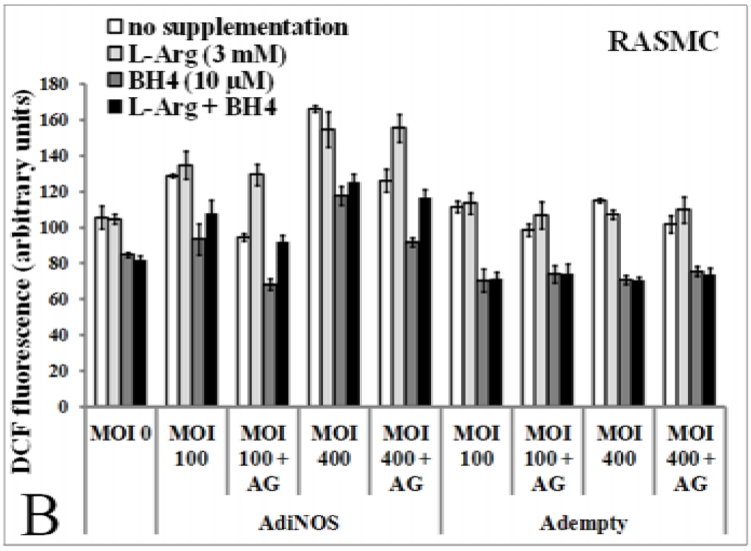
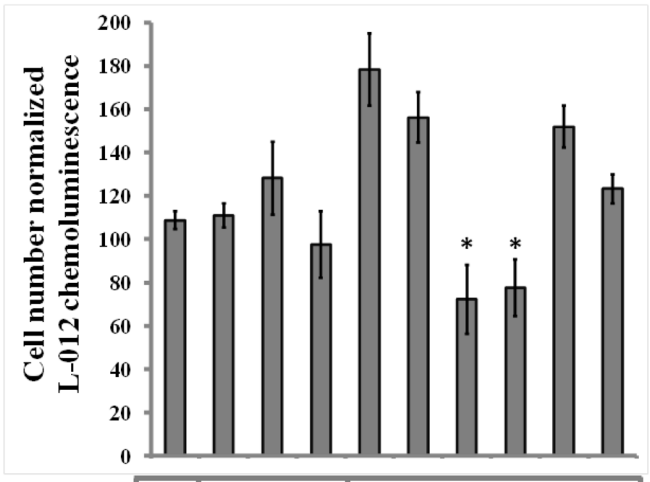


Figure 2. Effect of L-Arg and BH_4 levels on NO production from Ad_{iNOS} transduced RASMC and RAEC

Nitrite levels in conditioned cell culture media were determined by the Griess assay from untransduced or Ad_{iNOS} (100 MOI) transduced RASMC (A) or RAEC (B) after supplementation with increasing concentrations of BH_4 (0 – 10 μM) in the absence or presence of 3.0 mM L-Arg. Conversely, nitrite levels were measured in RASMC (C) or RAEC (D) after supplementation with increasing concentrations of L-Arg (0 – 3.0 mM) in the absence or presence of 10 μM BH_4 in standard media devoid of L-Arg. The monomeric/dimeric state of iNOS was determined by low temperature non-denaturing Western blot after Ad_{iNOS} (100 MOI) transduced RASMC (E) or REAC (F) were supplemented with L-Arg (3.0 mM) and BH_4 (10 μM).

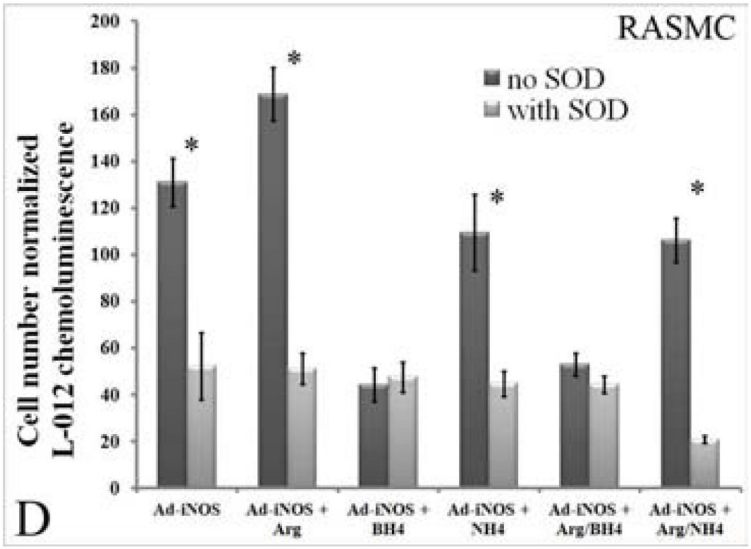






Transduction	none	Ad-empty			Ad-iNOS					
Supplementation	–	–	L-Arg NH4	L-Arg BH4	–	L-Arg	BH4	L-Arg BH4	NH4	L-Arg NH4

C



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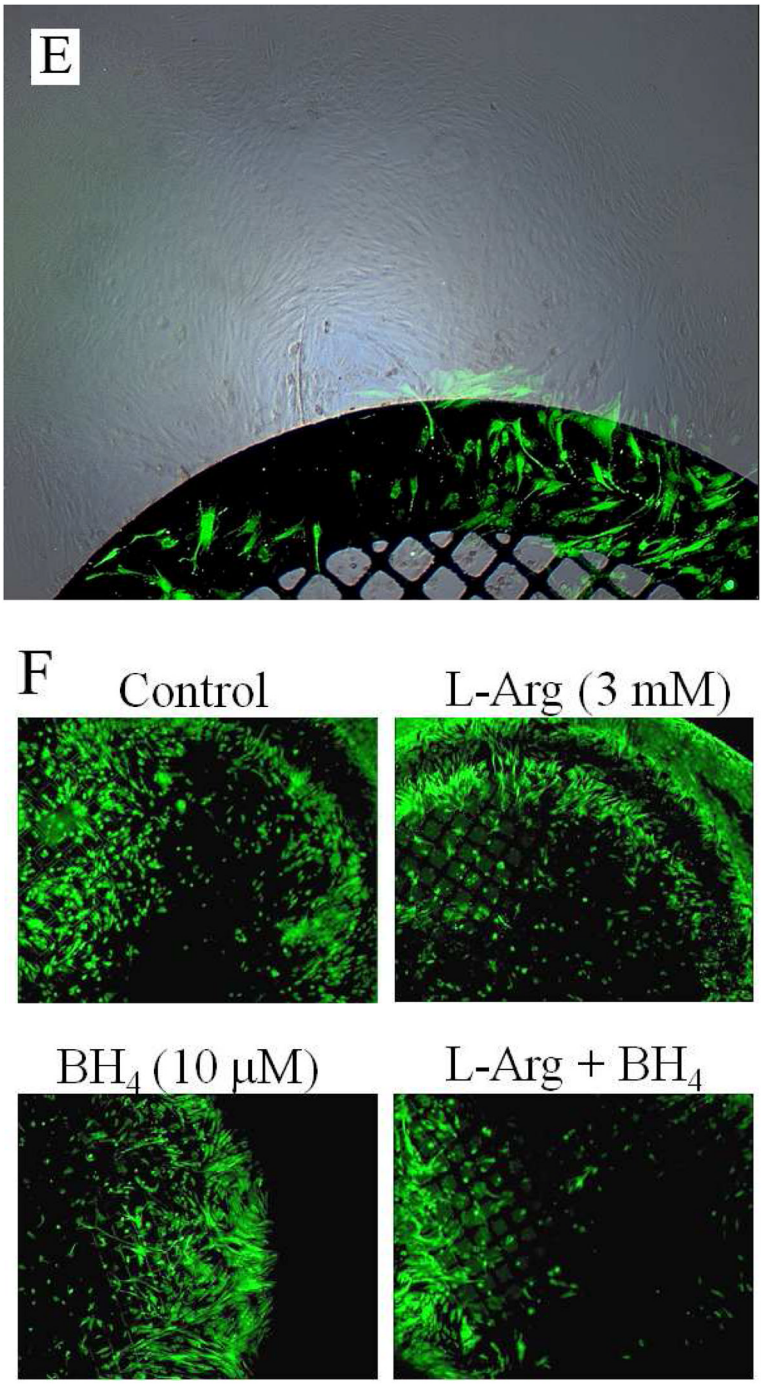
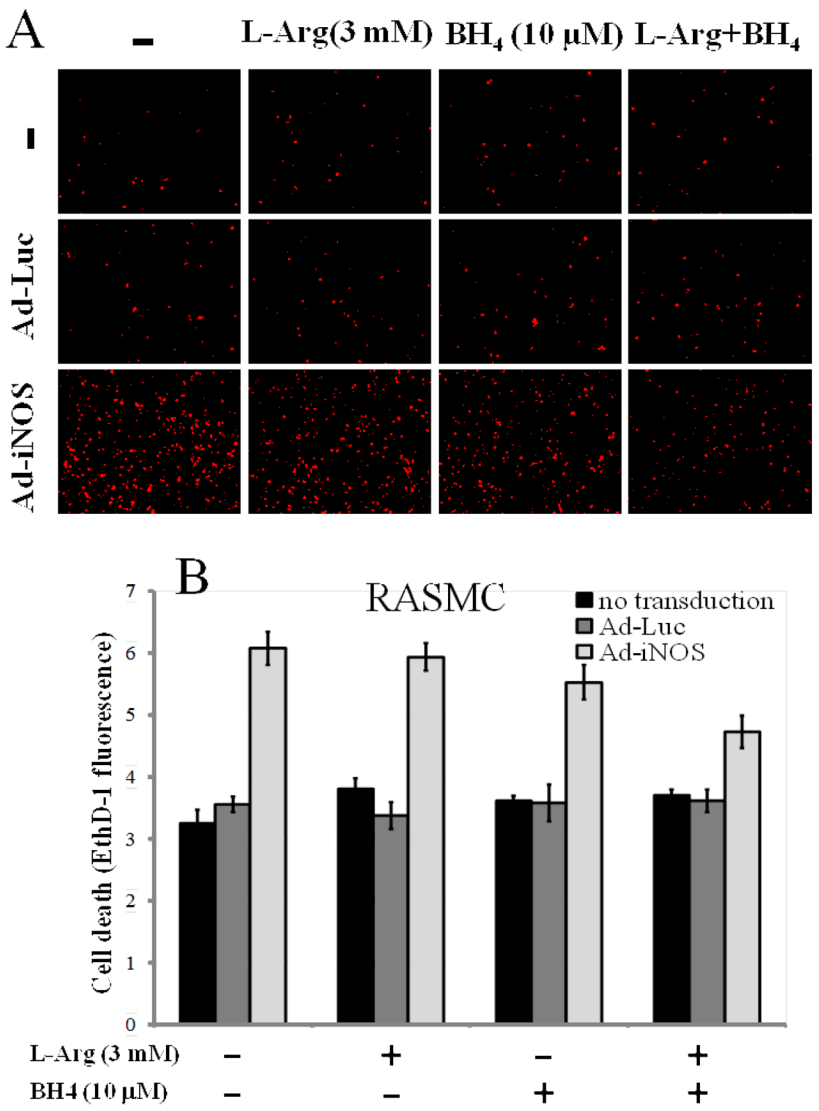
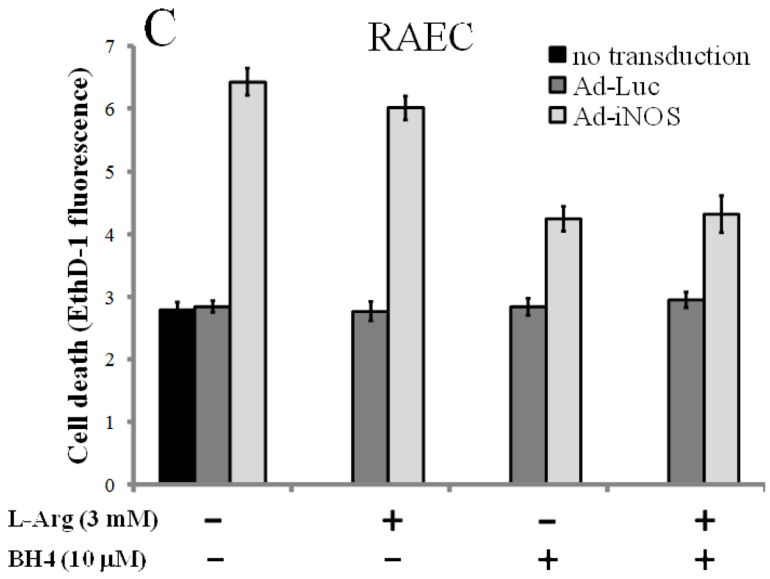


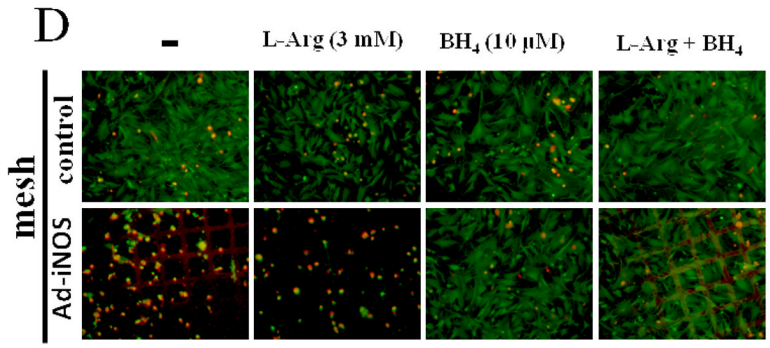
Figure 3. ROS production from Ad_{iNOS} transduced RASMC

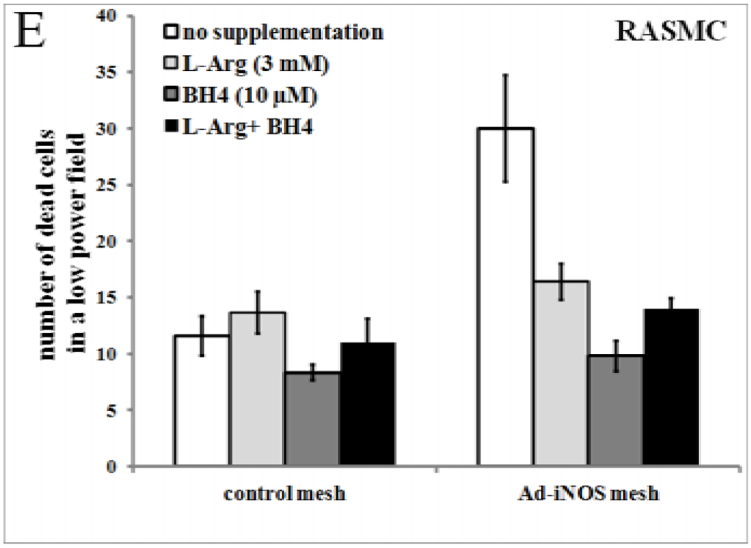
Primary RASMC were transduced with Ad_{iNOS} (0 – 600 MOI) and ROS production was measured 18 hrs later by DCF (3.0 μM) fluorescence (A). Primary RASMC were transduced with Ad_{empty} or Ad_{iNOS} (MOI 100 and 400) and supplemented with L-Arg/BH_4 18 hrs later in the absence or presence of 1.0 mM aminoguanidine and ROS production was measured 30 min after addition of L-Arg/BH_4 by DCF fluorescence (B). Primary RASMC were transduced with 400 MOI of Ad_{empty} or Ad_{iNOS} and supplemented with L-Arg (3.0 mM), BH_4 (10 μM) and NH_4 (10 μM) 18 hrs later. ROS were measured by L-012 (200 μM)

chemiluminescence and values are represented as means normalized to cell number ± SD. *, $p < 0.001$ vs. n0n-supplemented Ad_{iNOS} group (C). Primary RASMC were transduced with 400 MOI of Ad_{iNOS} and were treated with 100 U/mL of PEG-SOD followed by supplementation with L-Arg (3.0 mM), BH_4 (10 μM) and NH_4 (10 μM) 18 hrs post transduction. Results are represented as means of L-012 (200 μM) chemiluminescence normalized to cell number. *, $p < 0.001$ for the comparisons between the same type supplementation groups with or without PEG-SOD pretreatment (D). Overlay of bright field image and fluorescent DCF staining of primary RASMC after exposure to Ad_{iNOS} tethered mesh (E). Images of primary RASMC stained with DCF after exposure to Ad_{iNOS} tethered meshes and supplementation with L-Arg/BH_4 (F).









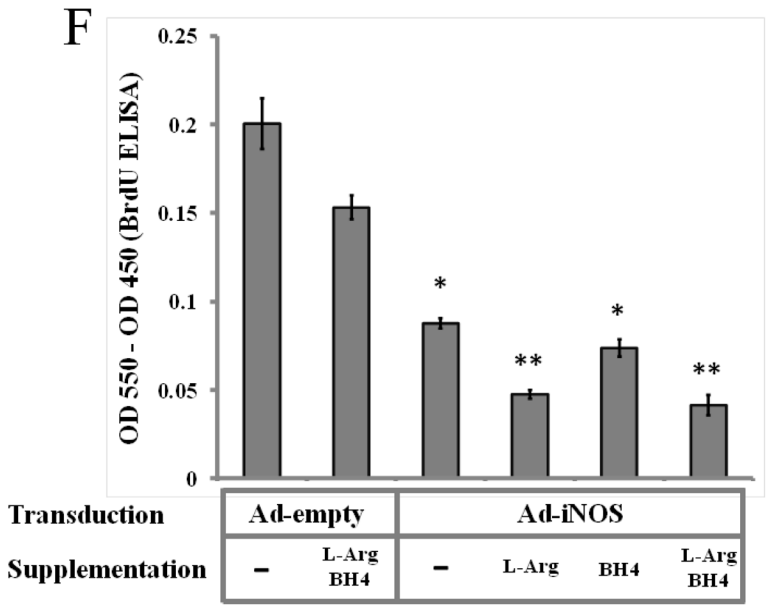


Figure 4. Effect of Ad_{iNOS} transduction on cell death and proliferation in RASMC and REAC

Primary RASMC were not transduced or were transduced with 400 MOI of Ad_{Luc} or Ad_{iNOS}. After 24 hrs of exposure to L-Arg/BH_4 cells were stained with EthD-1 (5.0 μM) and EthD-1 staining was evaluated by fluorescence microscopy (A) and fluorometry (B). Primary RAEC (C) were not transduced or were transduced with 400 MOI of Ad_{Luc} or Ad_{iNOS}. After 24 hrs of exposure to L-Arg/BH_4 cells were stained with EthD-1 (5.0 μM) and EthD-1 staining was measured by fluorometry. Primary RASMC were exposed to control, Ad_{Luc} tethered or Ad_{iNOS} tethered meshes. After 24 hrs of exposure to L-Arg/BH_4 cells were stained with EthD-1 (5.0 μM) and EthD-1 staining was evaluated by counting the number of dead cells from fluorescent microscopy images (D) and results were represented as the means ± SD (E). Primary RASMC were transduced with 400 MOI of Ad_{empty} or Ad_{iNOS} and supplemented with L-Arg (3.0 mM) and BH_4 (10 μM). Proliferation was measured by BrdU incorporation and results represent the mean of OD 550 – OD 450 ± SD. *, $p < 0.001$ vs Ad_{empty} and Ad_{empty} with L-Arg and BH_4. **, $p < 0.001$ vs Ad_{empty}, Ad_{empty} with L-Arg/BH_4 and Ad_{iNOS} (F).

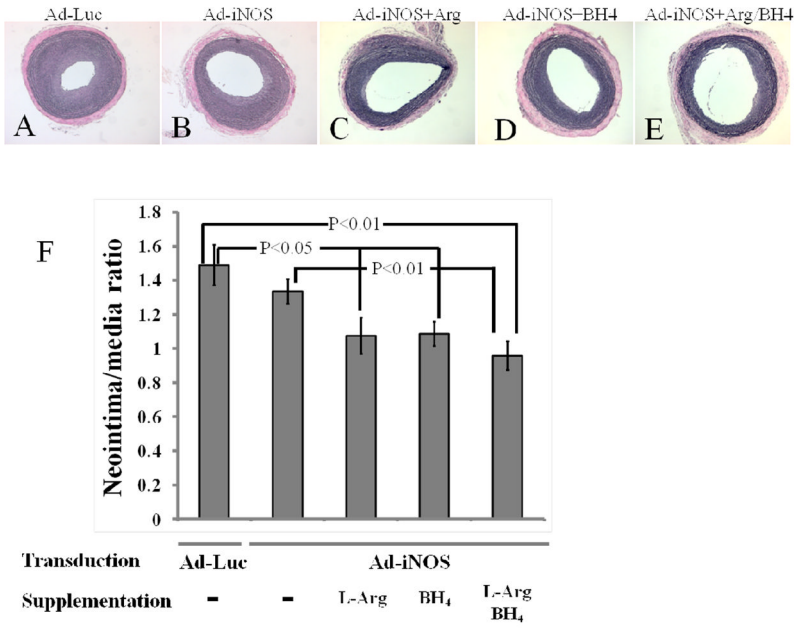


Figure 5. Effect of L-Arg and BH_4 supplementation on neointima formation in Ad_{iNOS} transduced rat carotid arteries

Ad_{iNOS} was delivered intraluminally to the balloon denuded carotid artery of male Sprague-Dawley rats. Rats were supplemented with 250 mg/kg/day L-Arg and 10 mg/kg/day BH_4 with 0.04% ascorbic acid in their drinking water for two days prior to surgery and 15 days post-surgery. Representative arteries stained with Verhoeff Van Gieson at 40 X magnification (A – E). Results represent the mean neointima/media ratio ± SEM (F). n = 12.