Adenoviral gene transfer of nitric oxide synthase: High level expression in human vascular cells

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Abstract

Objectives: Nitric oxide synthases (NOS) generate nitric oxide (NO), a second messenger with key regulatory roles. In the cardiovascular system, deficient endothelial NO production is an early, persistent feature of atherosclerosis and vascular injury. Accordingly, the NOS isoforms represent attractive targets for vascular gene therapy. We aimed to generate and evaluate an adenoviral vector for gene transfer of an NOS isoform to vascular cells. Methods: We constructed a recombinant adenovirus, Ad.nNOS, for gene transfer of the neuronal isoform of NOS (nNOS) and characterized its expression in 293 cells, human vascular smooth muscle cells (hVSMC) and human umbilical vein endothelial cells (HUVEC). NOS expression was analyzed by Western immunoblotting, and NOS enzyme activity in response to receptor-dependent and receptor-independent agonists was determined by Griess assay or by NO chemiluminescence. Results: Ad.nNOS-infected 293 cells expressed high levels of functional nNOS enzyme, even higher than in 293.NOS cells (a cell line that expresses supraphysiologic levels of nNOS). In hVSMC, nNOS activity reached levels 50% of those seen in 293.NOS cells. nNOS expression and activity in hVSMC increased linearly with titer of Ad.nNOS. NO production in hVSMC was stimulated both by calcium ionophore and by physiologic agonists such as acetylcholine or bradykinin. In HUVEC, endogenous NOS activity was significantly augmented by Ad.nNOS infection. Supplementation with the tetrahydrobiopterin precursor sepiapterin enhanced NOS activity in all cells. Conclusions: Ad.nNOS, a novel adenoviral vector for gene transfer of NOS, generates high-level nNOS expression in a variety of vascular cell types. nNOS activity in hVSMC is physiologically regulated and of a magnitude comparable to native eNOS activity in HUVEC. Our findings demonstrate Ad.nNOS to be a versatile and efficient tool for nNOS gene transfer, with widespread potential applications in cell culture and for gene therapy.

Keywords: Human, smooth muscle cell; Nitric oxide; Gene therapy; Gene expression; Gene transfer, Adenovirus; Human, umbilical vein endothelial cell (HUVEC)

1. Introduction

Gene transfer vectors derived from adenovirus have proven to be extremely effective for expression of heterologous genes [1]. Their advantages include a broad host range, the ability to obtain high transduction efficiencies in non-replicating cells and the ease of preparation of pure, high-titer stocks. These properties make adenoviral vectors suitable for gene therapy applications, especially for local gene delivery in the cardiovascular system [2-4]. Recombinant adenoviral vectors have been widely used to transfer marker genes to vascular cells and tissues, both in vitro and in vivo [5-7]; moreover, there have been increasing reports of their use to express functional proteins of biological significance [8-11].

Nitric oxide synthases (NOS) generate the free radical second messenger, nitric oxide [12,13]. Nitric oxide synthases exist in three distinct isoforms. The neuronal-type (nNOS) and vascular endothelial cell-type (eNOS) are primarily regulated by reversible binding of calcium-calmodulin. The third isoform, iNOS, is induced in a
variety of cell types by cytokine stimulation. It contains a tightly bound calmodulin subunit and remains active even at resting levels of intracellular calcium. All three isoenzymes catalyze the five-electron oxidation of L-arginine to L-citrulline. The oxidation requires exogenous NADPH, which binds to the enzyme, transfers electrons through the flavins FAD and FMN, to a heme group and thence to L-arginine. Tetrahydrobiopterin (BH4) maintains the enzyme in its active homodimeric form, and is essential for activity [14,15].

Nitric oxide (NO) regulates a broad range of biologic functions [16] including vascular tone [17,18], neurotransmission, immuno-regulation, and microbial killing. In the cardiovascular system, loss of endothelial NO production is an early event in atherosclerosis and other forms of vascular injury [19]. Early deficiency of endothelial NO production may in turn promote progression to more advanced vascular lesions since NO is an inhibitor of key processes in the pathogenesis of atherosclerosis, including platelet aggregation [20,21], leukocyte adhesion and smooth muscle cell proliferation [22,23]. Since NO plays a key role in the pathogenesis of vascular diseases, the nitric oxide synthases represent attractive targets for vascular gene transfer. The biologic utility of restoring eNOS activity in injured rat carotid arteries was demonstrated using plasmid–liposome gene transfer [24]. An adenoviral vector enabling higher-efficiency gene transfer of NOS would likely have wider therapeutic utility, both in vascular injury states where the endothelium is dysfunctional or lost, and in other organ systems. Accordingly, we aimed to develop an adenoviral vector for gene transfer of NOS.

We have recently developed a novel adenoviral vector system, based on the in340 strain of adenovirus type 5. It has E1 and E3 deletions, resulting in a cloning capacity of 7.5 kb, more than sufficient to accommodate the nitric oxide synthase cDNAs. In this report, we describe the use of this system to generate a recombinant adenoviral vector for gene transfer of the neuronal-type isoform of nitric oxide synthase (nNOS). We demonstrate both high level protein expression and functional nNOS enzyme activity in a variety of cell types, highlighting the potential utility of this novel vector for gene transfer and gene therapy applications.

2. Methods

2.1. Construction of recombinant Ad.nNOS

To generate Ad.nNOS we used a novel adenoviral vector system derived from the in340 mutant strain of adenovirus type 5 (Ad5) [25,26]. The in340 strain is distinguished by a duplicate E1a enhancer/viral packaging signal (Ad5 base pairs (bp) 194–351) at the right (3’) end of the virus. To enhance the utility of the in340 strain as a gene transfer vector, we deleted 2749 bp (Ad5 bp 28725–31000) from the E3 region, inserted a nuclear localizing β-galactosidase expression cassette in the E1 cloning region, and added three unique restriction sites (ClaI, XbaI and PacI) at the 3’ end of the E1 cloning site (Ad5 bp 3334). The resulting viral vector, Ad.Pac β Gal, served as the parental DNA for the recombinant vector system, based on the in340 strain of adenovirus type 5. It contains a β-galactosidase expression cassette in the E1 cloning region, and added three unique restriction sites (ClaI, XbaI and PacI) at the right (3’) end of the E1 cloning site (Ad5 bp 3334–3793). These fragments are located between the EcoRI and XhoI sites of the pGEM 4 plasmid (pGEM 4). The nNOS cDNA was cloned into pGEM CMV, a plasmid designed for generation of viral recombinants by either by ligation or by overlap recombination. pGEM CMV contains the adenovirus 5 (Ad5) inverted terminal repeat (Ad5 bp 1–194, ‘ITR’), a CMV early enhancer/promoter (‘CMV’), a cloning site for the insertion of a heterologous gene, and a portion of adenoviral DNA for overlap recombination (Ad5 bp 3335–3793). These fragments were located between the EcoRI and XhoI sites of the pGEM 4 plasmid (pGEM 4). The nNOS cDNA was cloned into the BamHI–XbaI sites of pGEM CMV, generating the plasmid pGEM CMV nNOS. This plasmid was modified by removing the 3’ non-coding region of the nNOS cDNA by digestion with NotI and XbaI, and replacing it with an SV40 VP1 polyadenylation signal (‘PA’), generating pGEM CMV nNOS PA. This plasmid was restriction digested with CiaI and Klenow DNA polymerase to create a blunt end. A 6 kb fragment, containing the left Ad5 ITR, CMV promoter, nNOS cDNA and SV40 PA, was generated by digestion of the linearized, blunted plasmid with XhoI. The DNA ‘backbone’ of the vector was prepared by XhoI digestion of genomic DNA from the parent vector, Ad.Pac β Gal, generating a 27 kb fragment with an ITR and the viral packaging signal (‘PKG’) at the extreme 5’ end. Both the 6 kb plasmid fragment and the 27 kb viral fragment were isolated from low-melt agarose gels by β-agarose digestion and ethanol precipitation. These fragments were ligated for 1 hour at room temperature using T4 DNA ligase. The ligated DNA was purified by phenol-chloroform extraction and ethanol precipitation and was transfected into 293 cells using the calcium phosphate method. Following observation of a cytopathic effect, recombinant Ad.nNOS was purified by plaque purification on 293 cells, as described in Section 2.
a control virus and was used to generate Ad.nNOS by replacing the \( \beta \)-galactosidase expression cassette with one containing an nNOS cDNA (Fig. 1). The cDNA for rat nNOS [27] was a generous gift from Dr. Solomon H. Snyder (Johns Hopkins University). This cDNA was cloned into the plasmid pGEM CMV and the 3' untranslated region of the nNOS cDNA was removed and replaced by the SV40 VP2 polyadenylation signal (pA). The resulting plasmid, pGEM CMV nNOS pA, was used to generate recombinant Ad.nNOS by direct ligation of the right 3/4 of the parent vector genome with a DNA segment from pGEM CMV nNOS pA (comprising the nNOS cDNA and SV 40 pA), the early CMV enhancer, the left terminal repeat of Ad5, and a segment of adenoviral DNA containing an engineered XbaI site, derived from the plasmid pGEM CMV). Details are provided in the legend to Fig. 1.

Viral DNA was purified from 293 cells infected with the parent vector Ad.Pac \( \beta \) Gal and was restriction digested with XbaI. The 27 kb viral DNA fragment was isolated from a low melt agarose gel using \( \beta \)-agarase digestion and ethanol precipitation. The plasmid pGEM CMV nNOS pA was restriction digested with XbaI and ClaI, and the 6 kb fragment was isolated and purified. The 6 kb plasmid fragment and the 27 kb viral fragment were ligated in vitro using T4 DNA ligase at room temperature for 1 h. The ligation mixture was purified using phenol-chloroform extraction and ethanol precipitation, then transfected into 293 cells using the calcium phosphate method. After observation of a cytopathic effect (approximately 5–7 days), the cells were lysed by multiple freeze cell cycles and recombinant virus was isolated by plaque assay on 293 cells. Plaques expressing high levels of enzymatically active NOS as judged by Griess assay [28] were isolated by two rounds of plaque purification [29].

### 2.2. Purification of high titer Ad.nNOS stock

Using plaque purified Ad.nNOS described above, we generated high titer stock by infecting forty 150-mm plates of confluent 293 cells, at a multiplicity of infection of 1, in DMEM/2% FBS. After observation of a cytopathic effect, cells were harvested and virus was purified on a cesium chloride gradient, using modifications of existing methods [29]. Briefly, the 293 cell pellet was lysed by Dounce homogenization, cell debris was pelleted, and the supernatant was adjusted to a density of 1.1 g·ml\(^{-1}\) by the addition of solid cesium chloride. This solution was layered onto a 1.3 g·ml\(^{-1}\)-1.4 g·ml\(^{-1}\) cesium chloride step gradient and ultracentrifuged for 2.5 h at room temperature. The visible band of pure virus was harvested and desalted by serial gel filtration on Sepharose CL-6B spin columns (Pharmacia, Uppsala, Sweden) in virus storage buffer (VSB; 20 mM Tris pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM MgCl\(_2\)). Gel filtered virus was diluted 1:1 with 80% fetal bovine serum (FBS)/20% glycerol and immediately frozen in aliquots. Titers of all viral stocks were determined by plaque assay on 293 cells using standard techniques [29].

### 2.3. Cell culture

Human vascular smooth muscle cells (hVSMC) were purchased from Clonetics Corporation (San Diego, CA), propagated and stored at \(-70^\circ\text{C}\) until further use. For experiments, cells were used in passages 5 to 9. VSMC exhibited typical 'hill and valley' morphology and stained with the HHF35 anti-smooth muscle actin monoclonal antibody. Human umbilical vein endothelial cells (HUVEC) and 293 cells were obtained from the American Tissue Culture Collection. 293 cells stably transfected with the rat nNOS gene (293.NOS cells) were a generous gift from Dr. Solomon H. Snyder [27]. Cells were cultured at 37°C, in a humidified 5% CO\(_2\) atmosphere, in either Dulbecco's Modified Eagle Medium (DMEM) (293 cells) or in Kaighn's F-12 Nutrient Mixture containing 10 IU·ml\(^{-1}\) heparin and endothelial cell growth supplement (Sigma, St. Louis, MO) (HUVEC). Media were from Gibco BRL (Bethesda, MD), contained 100 \( \mu \)g·ml\(^{-1}\) penicillin and streptomycin and were supplemented with 10% (v/v) fetal bovine serum (FBS) (Hyclone, Logan, UT). Human VSMC were cultured in DMEM supplemented with epidermal growth factor and fibroblast growth factor (Clonetics). During viral infection, serum concentration was reduced to 0.2% (293 cells) or was removed completely and replaced by 0.1% bovine serum albumin (hVSMC and HUVEC).

### 2.4. Infection of cultured cells with Ad.nNOS and determination of nNOS expression and activity

Cells were cultured to confluence in 6-well plates and infected for 1 h at 37°C with Ad.nNOS, using the titers indicated in the figures. Cells were cultured for an additional 24 h (in the case of 293 cells) or for 72 h (all other cell types) to allow protein expression. When the tetrahydrobiopterin precursor sepiapterin [30] (obtained from Dr. Schirks, Jonas, Switzerland) was used to increase cellular tetrahydrobiopterin levels, it was added to culture media at a concentration of 100 \( \mu \)M following viral infection. To determine NOS activity, cells were washed in phosphate-buffered saline (PBS), and incubated at 37°C in Krebs-Henseleit buffer (NaCl 120 mM, KCl 4.7 mM, CaCl\(_2\) 2.5 mM, MgSO\(_4\) 1.2 mM, KH\(_2\)PO\(_4\) 1.2 mM, NaHCO\(_3\) 25 mM, Glucose 5.5 mM, pH 7.4) containing 1 mM L-arginine and the calcium ionophore A23187 (Sigma, St. Louis, MO), at 1 \( \mu \)M. In some cases, the NOS inhibitor N-monomethyl-L-arginine (NMMA, Sigma) was added at 1 mM. After 6 h, the incubation buffer was assayed for nitrite and nitrate concentrations using the Griess assay [28]. In preliminary experiments, we measured both nitrate and nitrite production by first enzymatically reducing nitrate to nitrite using nitrate reductase. Nitrate levels were approximately 30% of nitrite levels. These proportions remained consistent
between experiments, and did not vary with total nitrite/nitrate levels. We therefore chose to measure nitrite alone to determine NOS activity, since prior dilution and incubation of samples for nitrate reduction reduced the overall sensitivity and precision of the Griess assay. NOS activity was expressed as the nmol of nitrite generated per mg of cellular protein per hour. The background level of nitrite generation in buffer incubated with control cells (0.5 – 1.0 nmol nitrite (mg protein)$^{-1}$·h$^{-1}$) was subtracted from all wells to give the net nitrite production. The lower limit of sensitivity of the Griess assay was approximately 0.5 nmol nitrite (mg protein)$^{-1}$·h$^{-1}$. In some experiments, we used physiologic agonists (acetylcholine (ACH) or bradykinin (BK)) or thapsigargin (TG) to stimulate nNOS activity in hVSMC. In these experiments, we used a shorter incubation period (1 h) and measured NO generation using a chemiluminescence method, which has a much greater sensitivity than the Griess reaction. NO was measured using a Nitrolite flow through system (Thermedics, Woburn, MA), following the reaction of NO with ozone in the Nitrolite system. Calibration was performed using standard known concentrations of GSNO, which gave a linear response over the range 50–1000 pmol GSNO. NOS activities in Ad.NOS-infected cells and control cells were compared using an unpaired Student’s t-test. A P value less than 0.05 was considered statistically significant.

2.5. NOS protein analysis by Western immunoblotting

After incubations to measure NOS activity, cells were harvested by scraping and pelleted by centrifugation. The cell pellet was resuspended in 150 μl ice-cold PBS containing phenylmethyl-sulfonylfluoride (0.2 mg · ml$^{-1}$) and leupeptin (0.5 μg · ml$^{-1}$), and lysed by sonication. Cell debris was pelleted and total protein concentration in the cell lysate was determined using the Bradford assay (Bio-Rad Laboratories, Richmond, CA). Equal samples (approximately 25 μg total protein) were separated by SDS PAGE on 9% polyacrylamide gels and electrophoretically transferred to a nitrocellulose membrane for Western analysis. The membrane was blocked in 5% non-fat milk and incubated with primary antibody for 1 h at room temperature. For detection of nNOS, eNOS and iNOS, isozyme-specific mouse monoclonal anti-NOS peptide antibodies were used at the manufacturer’s specified concentration (Transduction Laboratories, Lexington, KY). Protein bands were visualized using an anti-mouse IgG horseradish peroxidase-conjugated antibody followed by chemiluminescence. To quantify relative quantities of nNOS in cell lysates, serial dilutions of equal quantities of total protein were analyzed by Western blotting and bands were quantified by laser densitometry (LKB Ulotroscan XL). Relative protein quantity was expressed in arbitrary units. Linear regression, using the least squares method, was used to correlate enzyme activity with protein levels.

2.6. Staining of cells for NADPH-diaphorase activity

Following Ad.nNOS infection, hVSMC were stained for NADPH-diaphorase activity [32]. This stain demonstrates the presence of functional NOS protein. Briefly, hVSMC were cultured in 8-well microscope slide chambers (Lab Tek, Nunc, Naperville, IL) until sub-confluent. Cells were incubated for 72 h after Ad.nNOS infection to allow nNOS expression, then fixed for staining in 4% paraformaldehyde for 30 min. Cells were permeabilized for 30 min in 0.1 M Tris pH 7.2/0.2% Triton X-100, then stained with 1 mM NADPH, 0.2 mM nitroblue tetrazolium in the same buffer at 37°C for approximately 1 h, until the development of blue-purple staining was observed. Mock-infected cells were stained in parallel for the same period of time.

3. Results

3.1. Construction of a recombinant adenovirus for gene transfer of nNOS

Using a novel adenoviral vector system derived from Ad340, we constructed a recombinant adenovirus expressing nNOS. The construction is described in Fig. 1 and Section 2. Successful recombinants were selected on the basis of ionophore-stimulated nitrite production, and confirmed by analysis of viral DNA by restriction digestion.

3.2. Adenoviral mediated gene transfer of nNOS to cultured cells

3.2.1. 293 Cells

We evaluated the efficacy of Ad.nNOS gene transfer in 293 cells by comparing levels of nNOS protein and activity with those in a 293 cell line stably transfected with an nNOS expression plasmid. These cells, 293.NOS cells, are a well characterized source of supraphysiologic levels of nNOS and have been used to study nNOS function and regulation [27,33]. In 293.NOS cells, immunoreactive nNOS was readily detectable (Fig. 2). The 293.NOS cells generated high levels of nitrite after stimulation with calcium ionophore. Nitrite generation was enhanced by the presence of the BH4 precursor sepiapterin, and was inhibited by the arginine analog, N-mono-methyl-L-arginine (NMMA). In mock-infected 293 cells, immunoreactive nNOS protein was not detected and nitrite production was not significantly elevated over background. This was also
the case after infection with Ad.Pac β Gal, a marker virus encoding β-galactosidase (data not shown).

We compared these results with nNOS protein expression and enzyme activity in 293 cells infected with Ad.nNOS. We observed high-level nNOS expression and nitrite production after Ad.nNOS infection (Fig. 2). Laser densitometry of Western blots revealed 30% higher nNOS protein levels in Ad.nNOS infected cells, when compared to 293.NOS cells. NOS activity was also higher in Ad.nNOS infected cells than in 293.NOS cells. These findings show that Ad.nNOS infection generates levels of nNOS protein and activity in 293 cells, exceeding that found in 293.NOS cells.

3.2.2. Human vascular smooth muscle cells

We next sought to characterize nNOS gene transfer to human vascular smooth muscle cells (hVSMC). These cells were chosen because of their relevance to vascular gene therapy. Ad.nNOS infection resulted in nitrite production which was stimulated by calcium ionophore and inhibited by NMMA (Fig. 3). The level of NOS activity in
Ad.nNOS-infected hVSMC approached 50% of that observed in 293.NOS cells. Sepiapterin more than doubled NOS activity but had no effect on levels of immunoreactive nNOS protein. Western analysis revealed expression of nNOS in Ad.nNOS infected cells, but not in cells infected with a β-galactosidase encoding virus (Ad.Pac β Gal) or after mock infection. Adenoviral infection of hVSMC did not induce iNOS, as judged by Western analysis (data not shown). These findings were not specific for human cells; in rabbit primary VSMC, Ad.nNOS infection resulted in a maximum NOS activity of 5.5 nmol nitrite · (mg protein)−1 · h−1, and similar patterns of nNOS expression and activity were also observed in porcine VSMC, and in the rat A10 VSMC cell line.

To further demonstrate the presence of functional NOS, we stained hVSMC for NADPH-diaphorase activity [32]. Intense staining of the cytoplasm was seen in cells infected with Ad.nNOS, whereas control cells showed no staining (Fig. 4). This observation underscores the high levels of functional nNOS expressed in hVSMC following Ad.nNOS infection.

To investigate the relationship between nNOS protein expression and NOS activity, we evaluated the effect of infectious titer on these parameters. Both protein expres-

![Fig. 4. NADPH-diaphorase staining of human vascular smooth muscle cells following adenoviral gene transfer of nNOS. Human vascular smooth muscle cells were cultured to sub-confluence in 8-well microscope-slide culture wells plates (2 X 10^4 cells/well) and infected for 1 h in 0.1 ml medium containing 5 X 10^7 pfu·ml^-1 Ad.nNOS (multiplicity of infection 250) (panels B and C), or mock infection in medium alone (panel A). After 72 h incubation in growth medium with the addition of sepiapterin (100 μM), cells were fixed and stained for NADPH-diaphorase activity, as described in Section 2. Intense purple staining shows the presence of NADPH-diaphorase activity. Original magnification: panels A and B X 33, panel C X 100).](attachment://image.png)
sion and activity increased progressively with increasing titer of viral infection (Fig. 5); this relationship was linear up to a titer of $2 \times 10^8$ pfu \cdot ml$^{-1}$ ($r^2 = 0.95$, $P < 0.005$).

We next investigated the ability of physiologic agonists, known to stimulate NO production in vascular cells in vivo, to activate nNOS in hVSMC following Ad.nNOS infection. This question is important since it addresses the utility of Ad.nNOS for NO generation in physiologic settings. After Ad.nNOS infection, but not after Ad.Pac $\beta$ Gal infection or in uninfected cells, receptor-mediated NO generation was stimulated both by acetylcholine (ACh, 1

![Fig. 6. nNOS activity in human vascular smooth muscle cells stimulated by physiologic agonists.](https://academic.oup.com/cardiovascres/article-abstract/32/5/962/393652)

**Fig. 6.** nNOS activity in human vascular smooth muscle cells stimulated by physiologic agonists. Human vascular smooth muscle cells were cultured to confluence in 6-well culture plates ($3 \times 10^5$ cells/well) and infected for 1 h in 0.75 ml medium containing $5 \times 10^7$ pfu/ml-1 Ad.nNOS or Ad.Pac $\beta$ Gal (multiplicity of infection 100), or no virus. After incubation in growth medium for 3 days, with the addition of sepiapterin (100 $\mu$M), cells were stimulated for 1 h by the addition of acetylcholine (ACh, 1 $\mu$M), bradykinin (BK, 2 $\mu$M), thapsigargin (TG, 0.5 $\mu$M) or calcium ionophore (A23187, 1 $\mu$M) in Krebs-Henseleit buffer (KHB). NO and nitrite were measured in KHB using the Nitrolite chemiluminescence system; NOS activity is expressed as the quantity of nitrite produced per hour per mg of protein in the total cell lysate from each dish, following subtraction of background nitrite levels. Values represent the means of duplicate determinations ($n = 2$), and error bars represent standard deviations. Also shown is the statistical significance of the result compared with control cells ($\ast P < 0.05$, $\ast\ast P < 0.01$). The inset shows Western analysis of equal quantities of total cell lysate for nNOS or $\beta$ galactosidase ($\beta$ Gal) expression. The arrows (‘nNOS’ or ‘$\beta$ Gal’) show the positions of nNOS or $\beta$ Gal protein bands in positive control samples.

$\mu$M) and by bradykinin (BK, 2 $\mu$M) (Fig. 6). Levels of NOS activity stimulated by ACh or BK were approximately 40% or 50% respectively of the maximal activity stimulated by A23187. Receptor-independent nNOS activity could be stimulated not only by A23187 but also by thapsigargin (TG, 0.5 $\mu$M), which stimulates a rise in intracellular Ca$^{2+}$ by inhibiting intracellular Ca$^{2+}$-ATPase activity [31]. Use of the chemiluminescence method for determination of A23187-stimulated NOS activity gave very similar values to those obtained by Griess assay in
separate experiments (Fig. 3). As in previous experiments, nNOS protein expression was present equally in all cell dishes infected with Ad.nNOS, but was not seen in control cells or cells infected with Ad.Pac β Gal. Conversely, high level β-galactosidase expression was seen only in Ad.Pac β Gal infected hVSMC. These data demonstrate that nNOS expressed in hVSMC following Ad.nNOS gene transfer is regulated in a calcium-dependent manner by both receptor-dependent and receptor-independent agonists, analogous to native eNOS in endothelial cells [31]. Importantly, these findings highlight the potential utility of Ad.nNOS gene transfer for NO generation in in-vivo settings.

3.2.3. Human umbilical vein endothelial cells

In HUVEC, endogenous NOS activity (i.e., eNOS) was present that was stimulated by A23187 and inhibited by NMMA (Fig. 7). Infection with Ad.nNOS enhanced the total NOS activity by approximately 50%. Although sepiapterin had virtually no effect on eNOS activity, the additional NOS activity generated by Ad.nNOS infection depended entirely on the presence of sepiapterin. Western blotting revealed only eNOS in uninfected HUVEC, whereas in Ad.nNOS infected HUVEC, both eNOS and nNOS were present. Induction of iNOS was not detected in either uninfected HUVEC or in HUVEC following Ad.nNOS infection (data not shown). Thus, total NOS activity in HUVEC can be significantly augmented by Ad.nNOS-mediated gene transfer, again indicating the high-level expression obtained using Ad.nNOS.

4. Discussion

In this report we describe for the first time the construction of a recombinant adenovirus, Ad.nNOS, for gene transfer of nitric oxide synthase, a potentially important candidate gene for cardiovascular gene therapy. We show that Ad.nNOS infects a variety of cell types, including human vascular cells, and leads to expression of functional NOS at high levels.

4.1. Efficiency of nNOS gene transfer in cultured cells

Our studies of protein expression and enzyme activity in 293 cells document the effectiveness of Ad.nNOS for gene transfer in cultured cells. 293.NOS cells express about 10-fold higher levels of functional nNOS than cerebellar tissue [27]; nevertheless, Ad.nNOS-infected 293 cells meet or exceed the supraphysiologic NOS levels observed in 293.NOS cells. Since the 293.NOS cell line is a well-established high-level NOS expression system, these findings demonstrate the utility of Ad.nNOS as a novel system for NOS expression at even higher levels. Our adenoviral expression system also has the advantage of flexibility and versatility: we demonstrate high-level nNOS expression in a variety of cell types from different species. In addition, recombinant viruses based on Ad.nNOS could be used to express different NOS isoforms, or to express mutant NOS's for the study of NOS structure–function relationships or NOS regulation.

We also studied Ad.nNOS gene transfer in hVSMC and HUVEC, since vascular cells are more directly relevant to human gene therapy applications [4,9,34]. Adenoviruses have been widely used to infect these cell types with both marker and functional genes [5–11]. Ad.nNOS gene transfer again proved highly efficient. In hVSMC, Ad.nNOS infection results in nitrite levels about one-third of that observed in 293.NOS cells or in uninfected HUVEC. NOS activity was readily quantified using the simple but insen-
4.2 Modulation of NOS activity by sepiapterin

Although cytokine stimulation induces iNOS protein expression in VSMC, tetrahydrobiopterin (BH4) is an absolute requirement for NOS activity [30,39]. BH4 is undetectable in unstimulated cultured VSMC, but cytokine stimulation also rapidly upregulates GTP-cyclohydrolase (GTP CH; EC 3.5.4.16), the rate-limiting enzyme for de novo BH4 synthesis. Our data from Ad.nNOS-infected hVSMC are entirely consistent with these observations: enhancement of NOS activity above baseline requires supplementation with sepiapterin (a BH4 precursor which is converted to BH4 by the GTP-CH-independent pterin salvage pathway [30]). The observed sepiapterin requirement suggests that adenoviral gene transfer does not upregulate GTP-CH. These observations may have important implications for NOS gene therapy: in vivo enhancement of NO production may require an increase in BH4 levels. Co-infection with an adenovirus expressing GTP-CH would likely enhance BH4 synthesis, providing a potential solution to this problem. However, BH4 supplementation was not required for eNOS activity following in vivo gene transfer of eNOS by plasmid transfection, [24], suggesting that BH4 availability may be less limiting in vivo than in cultured cells.

4.3 Potential utility of nNOS gene transfer

Adenoviral gene transfer of NOS provides a new approach to investigating intracellular NOS regulation, and the cellular mechanisms underlying the pathogenesis of various diseases, such as neuronal degeneration, myocardial injury and atherosclerosis. In the cardiovascular system, NOS gene transfer represents a potentially important therapeutic strategy. Endothelial dysfunction is an early and consistent marker of vascular injury, and loss of nitric oxide production may facilitate atherosclerotic progression in a number of ways [40,41]. Accordingly, restoration of nitric oxide production by gene therapy may normalize vasomotor function, and may limit atherosclerotic progression. Such a beneficial effect of vascular NOS gene transfer was recently demonstrated in a denuded rat carotid artery model, using modified plasmid-liposome complexes [24]. An adenoviral approach offers potential advantages over liposome-mediated gene transfer, although the relative merits of each remains the focus of intense investigation [3].

Although eNOS is the only NOS isoform found in the normal vessel wall, it does not necessarily follow that eNOS will be superior to other NOS isoforms for vascular gene therapy. iNOS has the theoretical advantage of sustained high-level activity independent of agonist stimulation [12,13], but its effects may prove cytotoxic rather than beneficial [16]. In contrast, eNOS and nNOS are functionally similar; both are calmodulin-regulated, and produce NO transiently in response to agonist stimulation. Although eNOS is myristoylated and predominantly membrane associated, the functional similarities make it likely that gene-transferred nNOS could act as a surrogate for eNOS in the vascular wall. This is clearly illustrated by our demonstration that nNOS gene transfer significantly augments total NOS activity in HUVEC, a cell type that normally expresses only eNOS. Whereas eNOS gene transfer is one approach to restoring deficient vascular NO production, we hypothesized that the neuronal isoform of NOS would behave in a physiologic, calcium-dependent manner in a variety of other target cells (including vascular...
smooth muscle cells). Finally, a practical advantage of using nNOS for experimental vascular gene transfer is that NOS protein expression resulting from gene-transfer can be specifically distinguished from native eNOS by isoform-specific Western analysis, as we showed in HUVEC.

In summary, we have generated a recombinant adenovirus, Ad.nNOS, encoding an NOS isoform. We demonstrate that Ad.nNOS generates high levels of nNOS protein in vascular cells and that the expressed NOS is functional in response to both receptor-independent stimulation and to receptor-mediated agonists. Ad.nNOS provides a novel tool for high-level transient expression of NOS in cultured vascular cells and may have applications for vascular gene therapy.

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