Expression and function of a recombinant endothelial nitric oxide synthase gene in porcine coronary arteries

David G. Cable a, Timothy O’Brien b, Iftikhar J. Kullo c, Robert S. Schwartz c, Hartzell V. Schaff d, Vincent J. Pompili e,*

a Cardiac Surgical Research Center, Mayo Clinic and Mayo Foundation, Rochester, MN, USA
b Division of Endocrinology, Mayo Clinic and Mayo Foundation, Rochester, MN, USA
c Division of Cardiovascular Disease, Mayo Clinic and Mayo Foundation, Rochester, MN, USA
d Section of Cardiovascular Surgery, Mayo Clinic and Mayo Foundation, Rochester, MN, USA
e Krannert Institute of Cardiology, Department of Medicine, Indiana University School of Medicine, Indianapolis, IN, USA

Received 6 February 1997; accepted 5 June 1997

Abstract

Objectives: Direct gene transfer of exogenous nitric oxide synthase, with the subsequent increase in nitric oxide production, could represent a potential therapeutic strategy in the treatment of vascular proliferative disorders. The goal of the present study was to determine if porcine coronary arteries could be transduced with an adenoviral vector encoding endothelial nitric oxide synthase (Ad.CMVeNOS) resulting in functional expression. Methods and Results: Segments of porcine right coronary artery were exposed for 1.9 ± 7.8 days to either replication-deficient adenovirus encoding bovine endothelial nitric oxide synthase Ad.CMVeNOS, 5 × 10^9 pfu/ml, or control adenovirus encoding Escherichia coli β-galactosidase Ad.CMVLacZ, 5 × 10^9 pfu/ml. Immunohistochemistry with a monoclonal antibody specific for nitric oxide synthase NOS demonstrated recombinant gene expression in both the endothelial and adventitial layers of Ad.CMVeNOS transduced coronaries with only endogenous NOS confirmed in the endothelium of Ad.CMVLacZ arteries. Coronary arteries transduced with Ad.CMVeNOS yielded 517 ± 110 mean ± S.E.M. ng nitrite while vessels transduced with Ad.CMVLacZ yielded 126 ± 84 ng (P < 0.05, n = 6). Isometric tension recording, following prostaglandin F2α constriction, documented a reduced tension in Ad.CMVeNOS transduced coronaries, compared to matched Ad.CMVLacZ coronaries (6.10 ± 0.84 g vs. 8.45 ± 1.49 g, respectively, P = 0.05, n = 8). This tension differential was eliminated with prior incubation in Nω-monomethyl-L-arginine (L-NMMA, 10^{-5} M). The EC50 for calcium ionophore relaxation of Ad.CMVeNOS coronary arteries was reduced compared to Ad.CMVLacZ (−7.90 ± 0.03 logM vs. −7.26 ± 0.11 logM, respectively, P < 0.05, n = 8). Conclusions: These studies demonstrate successful transfer of endothelial nitric oxide synthase into porcine coronary arteries as verified by histochemical localization of recombinant protein with an increase of nitric oxide release as demonstrated by enhanced nitrite production and an alteration in vasomotor function. © 1997 Elsevier Science B.V.

Keywords: Nitric oxide synthase; Adenoviral vector; Gene therapy; Porcine coronary artery; Vasomotor function

1. Introduction

Three different genes encoding endothelial [1,2], neuronal [3,4], and macrophage nitric oxide synthase [5,6] have been cloned, illustrating that diverse processes such as vascular signaling, neurotransmission in the brain and cell-mediated toxicity are dependent on the production of nitric oxide. In the cardiovascular system, nitric oxide is a potent vasodilator and plays a key role in the modulation of vascular tone. In addition, nitric oxide: (a) inhibits smooth muscle cell proliferation and migration, (b) inhibits platelet aggregation, (c) inhibits leukocyte adhesion, and (d) scavenges superoxide anions [7–10].
Successful direct gene transfer of the endothelial nitric oxide synthase gene into injured rat carotid arteries has been demonstrated to increase vasomotor relaxation and inhibit intimal hyperplasia [11]. These results suggest that direct gene transfer with subsequent increase in nitric oxide production could represent a potential therapeutic strategy in the treatment of cardiovascular disease. However, the rat carotid injury model has limitations as a model of human vascular proliferative diseases such as coronary restenosis following revascularization procedures. Coronary arteries of pigs respond to vascular injury analogous to humans [12]. We therefore have proceeded with the development of adeno viral gene transfer into porcine coronary arteries to study the biological effects of recombinant nitric oxide synthase expression.

The purpose of the present investigation was to demonstrate the functional expression of recombinant nitric oxide synthase in porcine coronary arteries.

2. Methods

Procedures and handling of transduced tissues were reviewed and approved by the Institutional Biosafety Committee of the Mayo Foundation in compliance with the Guidelines for Research Involving Recombinant DNA Molecules published by the National Institute of Health (NIH Publication No. 59FR34496, amended 1995). Procedures and handling of animals were reviewed and approved by the Institutional Animal Care and Use Committee of the Mayo Foundation in compliance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH Publication No. 80-23, revised 1978).

2.1. Generation of adeno viral vectors

The cDNA for bovine endothelial nitric oxide synthase (eNOS) was provided by Dr. David Harrison at Emory University (Atlanta, GA). The plasmid vector, pACCMVpLpA, was provided by Dr. Robert Gerard at the University of Texas Southwestern Medical Center (Dallas, TX). The eNOS cDNA was ligated, using EcoRI and HindIII sites, and cloned into pACCMVpLpA. The ensuing plasmid was linearized with NruI. Cotransfection with aden viral dl309 into the 293 cell line was performed with calcium phosphate/DNA coprecipitation. dl309 is a restriction enzyme-site-loss variant of wild type adenovirus type 5 which retains only a single XbaI site at nucleotide 1339 [13]. 293 cells are human embryonic kidney carcinoma cells which have been transformed with the left end of human adenovirus type 5 DNA [14]. Recombinant adenoviral vectors were generated by homologous recombination [15]. Viral plaques were isolated and propagated in 293 cells. Enrichment of viral DNA by the Hirt extraction [16] and screening by restriction mapping and PCR documented the presence of eNOS cDNA (Ad.CMVeNOS). Positive plaques underwent two further rounds of plaque purification in 293 cells. Stocks were prepared from positive plaques and used to generate high titer preparations. The subsequent viral titer was determined by the plaque assay [17]. Adenovirus encoding Escherichia coli β-galactosidase (Ad.CMVLaCZ), used in all experiments as a control, was a gift of Dr. James Wilson of the University of Pennsylvania (Philadelphia, PA). It was propagated, isolated, and quantified as described above for Ad.CMVeNOS.

2.2. Ex vivo gene transduction

Segments, 4 mm in length, of freshly prepared porcine coronary arteries were removed from euthanized pigs. The coronary arteries were then immersed in cold (4°C), oxygenated physiological salt solution of the following composition (mM): NaCl, 118.3; KCl, 4.7; MgSO₄, 1.2; KH₂PO₄, 1.22; CaCl₂, 2.5; NaHCO₃, 25.0; and glucose, 2.2. The time delay between excision of tissue and ex vivo transduction was less than 30 min. The arterial segments were transduced with either Ad.CMVeNOS or Ad.CMVLaCZ at a titer of 5 × 10⁹ plaque forming units/ml in diluted MEM at 37°C. The arterial segments were then transferred to fresh medium 199 and incubated for 48 h at 37°C in a 5% CO₂ incubator (Forma Scientific, Inc., Marietta, OH).

2.3. Histochemistry

Histochemical staining of vessels for β-galactosidase activity was initiated with fixation of segments in 2% paraformaldehyde/0.2% glutaraldehyde in phosphate buffered saline (PBS) for 30 min. The vessels were rinsed twice with PBS and placed in 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) reagent at 37°C for 1.5–4 h, the staining was terminated by return to fixation solution prior to nonspecific staining of control specimens [18]. Vessels were then dehydrated through graded alcohol to xylene washes and, subsequently, embedded in paraffin. Serial, 5 μm every 50 μm, sections were counterstained with eosin.

Immunohistochemical staining of vessels for eNOS expression was initiated with flash freezing (−70°C) of artery segments in O.C.T. compound (Miles, Inc., Elkhart, IN). Serial, 5 μm every 50 μm, sections were obtained and immersed in acetone (4°C) and 1% paraformaldehyde/EDTA. The sections were then incubated in 0.1% sodium azide/0.3% hydrogen peroxide and incubated in 5% goat serum/PBS-Tween 20 to complex non-specific protein binding locales. An eNOS monoclonal antibody (5 μg/ml, 1:50 dilution, Transduction Laboratory, Lexing-
ton, KY) was applied for 60 min at room temperature, followed by incubations with biotinylated rabbit anti-mouse F(ab')2 (1 : 300, 20 min) secondary antibody and peroxidase-conjugated streptavidin (1 : 300, 20 min, Vector Laboratories, Burlingame, CA). Immunoreactivity of eNOS was documented by 3-amino-9-ethylcarbazole and hematoxylin counterstaining following 0.1 mol/l sodium acetate buffer (pH 5.2) immersion for 30 s. The specificity of eNOS immunolabelling was controlled by omission of primary anti-goat antiserum in the incubation medium in random sections.

2.4. Measurement of nitrite production

Nitrite measurements were made from 1 ml aliquots of Krebs’ buffer with 10^-4 M L-arginine and 10^-6 M calcium ionophore A23187 in which coronary arteries transduced with either Ad.CMVeNOS or Ad.CMVLaClZ were incubated for 2 h at 37°C. Nitrite production was measured using a spectrofluorometric assay [19]. This technique is based upon the chemical reaction of 2,3-diamino-phthalene (DAN) with nitrite which, under acidic conditions, yields the fluorescent product 1-(H)-naphthotriazole. One hundred microliters of freshly prepared DAN (0.05 mg/ml in 0.62 M HCl) was added to 1 ml of sample. After a 10 min incubation at 20°C, the reaction was terminated with 50 μl of 2.8 N NaOH. The addition of base maximized the fluorescent signal produced by the product. Sodium nitrite standards are made fresh in Type I H2O. The intensity of fluorescence was measured in a System 3 Scanning Spectrofluorometer (Farrand Optical Co., Vahalla, NY) with excitation at 365 nm and emission at 450 nm using a 1.5 ml disposable polystyrene cuvette.

2.5. Endothelium-dependent relaxations to calcium ionophore A23187

Segments of transduced coronary segments were evaluated in parallel in organ chambers (25 ml) containing Krebs’ solution gassed with 95% O2 and 5% CO2 (pH 7.4, 37°C). Segments were suspended by two stainless steel stirrups mounted to a strain gauge for continuous isometric force measurement (Statham UC2, Gould, Inc., Cleveland, OH). The optimal point of the length–tension relation was obtained by progressive stretching until contraction with KCl (20 mmol/l) at each sequential stretch was maximal [20,21]. The functional integrity of endothelium was tested by the presence of relaxations to bradykinin (10^-8 M). After optimal tension was attained, the segments were allowed to equilibrate for 30 min in the presence of the cyclooxygenase inhibitor indomethacin (10^-5 M) to inhibit endogenous prostanoid production. Relaxations to calcium ionophore A23187 (10^-9–10^-6 M) were obtained during contractions to prostaglandin F2α (2 × 10^-6 M). At the conclusion of the dose–response curve, papaverine (3 × 10^-4 M) was administered to determine absolute maximal relaxation and defined as 100% relaxation. In a separate group of transduced coronary arteries, segments were allowed to equilibrate for 45 min in the presence of the cyclooxygenase inhibitor indomethacin (10^-5 M) and the nitric oxide synthase inhibitor N^6-monomethyl-L-arginine (L-NMMA, 10^-4 M), relaxations to calcium ionophore A23187 (10^-9–10^-6 M) were obtained during contractions to prostaglandin F2α (2 × 10^-6 M).

2.6. Drugs

The drugs, L-arginine, L-NMMA, indomethacin, prostaglandin F2α, and calcium ionophore A23187, were obtained from Sigma Chemical Co., St. Louis, MO. All drugs were prepared in double-deionized distilled water except for indomethacin, dissolved in Na2CO3 (1 × 10^-5 M) and calcium ionophore A23187, dissolved in DMSO (8 × 10^-3 M final concentration) and further diluted with distilled water. This final concentration of DMSO does not alter smooth muscle function [22,23]. The concentrations are expressed herein as final molar concentration in the bath solution.

2.7. Statistical analysis

Results are expressed as mean ± standard error of the mean (S.E.M.). In all experiments, ‘n’ refers to the number of porcine coronary arterial segments taken. Only paired segments, treated with either Ad.CMVeNOS or Ad.CMVLaClZ, are reported herein. Statistical evaluation of data was performed with Student’s t-test for paired observations. Two-tailed analysis of variance (ANOVA) with post-hoc Newman-Keuls was performed on the dose–response curves to calcium ionophore. Values were considered to be statistically significant when P < 0.05.

3. Results

3.1. Localization of reporter gene

Following 48 h of ex vivo incubation, segments of porcine coronary arteries were stained en face for β-galactosidase activity in every experiment (n = 14). Luminal and adventitial staining was noted in coronaries transduced with Ad.CMVLaClZ only, no β-galactosidase staining was noted in paired coronaries transduced with Ad.CMVeNOS (Fig. 1A and B). Histochemical analysis of these sections confirmed localization of recombinant β-galactosidase activity in luminal endothelial cells and adventitial fibroblasts (Fig. 1C and D) in Ad.CMVLaClZ coronaries.

3.2. Localization of nitric oxide synthase

Transduced coronary arteries were also subject to immunohistochemical staining with a monoclonal antibody
specific for endothelial nitric oxide synthase. As with β-galactosidase staining, immunohistochemistry was performed after each viral transduction experiment. Arteries transduced with Ad.CMVlacZ demonstrated constitutive levels of NOS expression within the endothelium only (Fig. 1E). Arteries transduced with Ad.CMVeNOS demonstrated not only endothelial expression of NOS but also marked expression of NOS within adventitial cells (Fig. 1F,G,H).

3.3. Nitrite production of porcine coronary arteries

Porcine coronary arteries were stimulated for 2 h with L-arginine and calcium ionophore at 37°C and total nitrite production was determined by spectrofluorometric analysis. Coronaries transduced with Ad.CMVlacZ yielded 126 ± 84 nM nitrite/mg protein (n = 6) while Ad.CMVeNOS transduced coronaries yielded 517 ± 110 nM nitrite/mg protein (Fig. 2).

3.4. Isometric tension recordings of vasomotor function

Precontraction with prostaglandin F₂α (2 × 10⁻⁶ M) generated reduced tension in Ad.CMVeNOS transduced coronaries, 6.10 ± 1.08 g versus 8.45 ± 1.19 g in Ad.CMVlacZ coronaries (P = 0.05, n = 8). This tension differential was eliminated with prior incubation in N⁵G-monomethyl-L-arginine (L-NMMA, 10⁻⁴ M), prostaglandin F₂α constriction produced equivalent tension was developed in Ad.CMVeNOS and Ad.CMVlacZ coronaries (8.08 ± 2.03 g vs. 9.40 ± 2.46 g, respectively, P = NS, n = 6). Porcine coronary arteries transduced with Ad.CMVeNOS demonstrated augmented relaxation to increasing concentrations of calcium ionophore in the presence of indomethacin (Fig. 3A). The concentration of calcium ionophore required to generate a relaxation equivalent to 50% maximum, EC₅₀, was calculated to be 7.90 ± 0.03 μM in Ad.CMVeNOS transduced coronaries and 7.26 ± 0.11 μM in Ad.CMVlacZ transduced coronaries.
ies ($P < 0.05, n = 8$). Pretreatment of transduced vessels with L-NMMA ($10^{-4}$ M) attenuated calcium ionophore-mediated relaxations (Fig. 3B).

4. Discussion

This investigation of porcine coronary arteries demonstrates (1) expression of recombinant genes as demonstrated by augmented immunohistochemical staining of NOS but absent histochemical staining of β-galactosidase activity, (2) enhanced nitrite production, (3) augmented response to increased calcium ionophore concentrations, and (4) attenuation of calcium ionophore-induced relaxations with competitive inhibition of nitric oxide synthase.

The demonstration of transgene expression by a monoclonal antibody specific for NOS is complicated by the constitutive nature of endogenous NOS in the coronary artery. Indeed, endothelial expression of NOS was documented in the porcine coronary arteries transduced with Ad.CMVLacZ. In light of the NOS expression present in both the endothelium and adventitia of Ad.CMVeNOS transduced coronaries, treated identically exclusive of the viral vector utilized, it is seductive to attribute the entirety of the adventitial localization of NOS in these coronaries to transgene expression. However, the selective recruitment of inducible NOS within Ad.CMVeNOS must be addressed. The complexity of isoform expression of NOS is only now reaching clarity. Indeed, constitutive expression of iNOS (type II) has been demonstrated in certain cell lines and tissues [24,25]. This is in contrast to the majority of the literature which documents iNOS only in response to cytokines or lipopolysaccharide [26–28]. Transcriptional elements receptive to cytokines, NF-κB/Rel, have been demonstrated upstream iNOS, both in animals and humans [29,30]. In confirmation of the constitutive expression of iNOS, low levels of iNOS promoter activity have been demonstrated in human tissue in the absence of cytokine stimulation [30]. Nonetheless, since cytokines are freely diffusible with the vascular wall, the selective expression of NOS within the endothelium and adventitia, but not the media, could be singularly interpreted as transgene and eNOS (type III) expression. Furthermore, the vector, at the viral titer utilized in the current investigation, has been demonstrated not to generate cytotoxicity or functional iNOS activity [31].

Further evidence of transgene expression is provided by analysis of stimulated nitrite production in transduced coronaries; Ad.CMVeNOS transduced coronary arteries generated fivefold more nitrite than Ad.CMVLacZ transduced arterial segments. Porcine coronary arteries transduced with Ad.CMVeNOS produced less tension with KCl than matched arteries transduced with Ad.CMVLacZ, a differential effect eliminated by a competitive inhibitor of NOS, L-NMMA. The $EC_{50}$ for calcium ionophore, in the presence of cyclooxygenase inhibition, was reduced by Ad.CMVeNOS transduction. In review, though each methodology to document augmentation of the nitric oxide synthase pathway by Ad.CMVeNOS transduction has specific limitations, the combined consensus of the present investigation clearly suggests recombinant nitric oxide synthase expression and, subsequent, function.

The presented results are in conformity with those reports by van der Leyen and colleagues [11]. The expression of recombinant eNOS in rat carotid arteries reduced KCl evoked contractions. The current investigation has been extrapolated to the coronary circulation of an animal model well characterized to the human coronary circulation.

Nitric oxide synthase was chosen as an candidate for adenoviral directed gene therapy as nitric oxide is capable of inhibiting platelet aggregation, smooth muscle proliferation, and leukocyte adhesion, in addition to serving as a superoxide scavenger. Though these actions of nitric oxide were not specifically addressed in the current ex vivo investigation, we have shown our adenoviral vector encoding for bovine endothelial nitric oxide synthase is capable of augmenting nitric oxide production in the coronary circulation.

Impairment of endothelial-dependent response to platelets, and the subsequent production of nitric oxide, has been demonstrated 4 weeks following endothelial denudement in a porcine model [32]. Similarly, in a canine model of coronary reperfusion injury, it has been demonstrated that endothelium-dependent relaxation to receptor-dependent agonists was significantly impaired through G-protein dysfunction and impaired generation of nitric oxide [33]. Reperfusion injury and subsequent oxygen radical endothelial injury has also been demonstrated to inhibit receptor-dependent release of nitric oxide and lead to endothelial dysfunction [34]. Clearly, augmentation of nitric oxide production by adenoviral-mediated transgene expression within these clinical scenarios would impart a degree of normalcy. The demonstration of beneficial action with this therapeutic intention will be the subject of future investigation.

These studies define the biological function of an adenoviral vector in an ex vivo porcine coronary model. Techniques for endovascular coronary delivery are continuing to be refined, including systems which yield efficient adventitial delivery with a transvascular injection catheter [35]. Alternatively, the coronary circulation is available for prolonged periods of time during surgical procedures. In our model, combined adventitial and luminal expression of recombinant Ad.CMVeNOS has led to beneficial biological responses and will merit further in vivo studies to define its therapeutic role in the treatment of vasculoproliferative diseases such as restenosis after percutaneous coronary angioplasty.

In conclusion, the present study demonstrates adenoviral-mediated expression of eNOS in porcine coronary ar-
teries resulting in enhanced immuno-reactivity, nitrite production, and relaxation to agonists of NOS.

Acknowledgements

Supported in part by The Mayo Foundation and the Herman C. Kranert Foundation. Performed during D.G.C.’s tenure as a Clinical Investigator Research Fellow.

The authors wish to thank Marilyn Oeltjen and Sharon Guy for devoted technical support, Steve Ziesmer for exact technical performance of the immunohistochemical staining and Zvonimir S. Katusic, M.D., Ph.D. for assistance with viral preparation and nitrite assay.

References

[15] Spector DJ, Samaniego LA. Construction and isolation of recombi-