eNOS gene delivery prevents hypertension and reduces renal failure and injury in rats with reduced renal mass

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Abstract

Background. Impaired nitric oxide (NO) release in chronic renal failure has been implicated in the pathogenesis of hypertension and the progression of renal insufficiency. We investigated whether gene delivery of the endothelial NO synthase (eNOS) improves NO release and reduces blood pressure and renal failure and injury in rats with reduced renal mass.

Methods. Renal failure was induced by renal artery branches ligation. Two weeks later, rats with renal failure were divided into three groups and received an intravenous injection of the vehicle or the adenovirus that expresses eNOS or β-galactosidase (β-gal). Systolic blood pressure, renal parameters and histopathology were assessed at Week 4 after gene delivery.

Results. At the end of the study, systolic blood pressures, serum creatinine, proteinuria, urinary endothelin-1 (ET-1) excretion and renal cortex ET-1 levels were increased, whereas plasma and urine NO2/NO3 were reduced in renal failure rats as compared to normal controls. Renal injury comprised blood vessel media hypertrophy, focal and segmental glomerular sclerosis, tubular atrophy and interstitial fibrosis. Gene delivery of eNOS, but not β-gal, prevented an increase in systolic blood pressure and proteinuria, and a reduction in plasma and urine NO2/NO3. eNOS gene delivery also reduced a rise in serum creatinine, urinary ET-1 excretion and renal cortex ET-1 levels, and the renal vascular, glomerular and tubular injury.

Conclusion. This study indicates that eNOS gene delivery in rats with renal failure improves NO release, which likely prevents the aggravation of hypertension and slows down the progression of renal failure and injury.

Keywords: adenovirus; eNOS; endothelin-1; hypertension; renal failure

Introduction

Endothelial dysfunction is associated with diseases that have a high risk of cardiovascular morbidity and mortality including hypertension, heart failure, arteriosclerosis, diabetes and chronic kidney disease. In patients with chronic renal failure, endothelial dysfunction is characterized by a reduction in the endothelium-dependent vasorelaxation response to acetylcholine due to reduced endothelial nitric oxide (NO) release [1]. This reduction in NO release will likely potentiate the vasoconstrictor effect of neurohumoral factors, such as catecholamines, angiotensin II (Ang II) and endothelin-1 (ET-1), that increase total peripheral vascular resistance [2, 3]. Moreover, the action of these and other growth factors on vascular smooth muscle cells is unopposed promoting thereby vascular remodeling and media hypertrophy. In the kidney, inappropriate NO release is associated with altered renal hemodynamics, increased glomerular capillary pressure and sclerosis, tubular atrophy and interstitial fibrosis [4]. In keeping with these observations, mice lacking the endothelial NO synthase (eNOS) develop hypertension and vascular hypertrophy [5]. In addition, chronic inhibition of NO synthesis with the L-arginine analog Nω-nitro-L-arginine methyl ester (L-NAME) causes severe hypertension associated with glomerular injury and proteinuria [6–8], further supporting a pivotal role for basal NO release. Hence, the impairment of NO release in diseases such as chronic renal failure may lead to major cardiovascular and renal abnormalities.

The mechanism underlying the reduction of NO release in chronic renal failure still remains elusive. However, this has been related, at least in part, to a reduction in the NOS substrate L-arginine and cofactors’ availability and/or NOS activity and expression [9]. For instance, we and others documented that dietary supplementation with L-arginine reduces the aggravation of hypertension and the progression of renal failure in rats with reduced renal mass that are associated with an increase in NO availability and a reduction in ET-1 production [10]. However, it has been...
postulated that the protective effect of l-arginine could be due, in part, to an increase in agmatine generation, an endogenous non-catecholamine ligand for alpha-2 adrenoceptors and imidazole receptors produced by arginine decarboxylase that has cardiovascular beneficial properties [11]. Similar to l-arginine supplementation, vascular and renal protection has been reported with tetrahydrobiopterin (BH4) supplementation, a critical cofactor for eNOS activity [12]. In the presence of inappropriate concentrations of BH4, eNOS is uncoupled and produces NO but also superoxide anions that inactivate NO. Indeed, NO and superoxide anions are highly reactive molecules that form peroxynitrite, a powerful oxidant molecule [13]. In renal failure conditions, superoxide anion generation is also induced by factors such as Ang II that may further limit NO availability [14]. On the other hand, impaired NO release in patients with renal failure has been related to an accumulation in plasma of endogenous methylated analogs of L-arginine such as asymmetric dimethylarginine (ADMA), which inhibits NOS activity similar to L-NAME [15]. Finally, Vaziri et al. [16] reported that eNOS expression and activity are significantly lower in vascular and renal tissues of rats with chronic renal failure. The reduction in renal eNOS expression, but also in neuronal and inducible NOS isoforms, may, however, account for the reduction in renal NO release [17]. In contrast, others found an increase in eNOS expression in the aorta [18].

The present study was designed to investigate whether eNOS gene delivery using a recombinant replication-deficient adenovirus increases NO release in rats with renal failure and results in an improvement of hypertension and decreases the progression of renal failure and injury.

Materials and methods

Adenovirus vectors

The replication-deficient adenovirus containing the bovine aortic endothelial cell eNOS messenger RNA (mRNA) sequence under the control of cytomegalovirus (CMV) promoter was generated by group of William Sessa [19] and generously provided by Jean-Philippe Gratton (CRIM, Montreal, Quebec, Canada). This adenovirus also contains in tandem the enhanced green fluorescent protein (GFP), which was used to assess gene delivery efficacy. The adenovirus containing the β-galactosidase (β-gal) gene was used as a control (gift of Bruce C. Trapnell, Genetic Therapy Inc, MD) [20]. These adenoviruses were amplified in HEK-293 cells and purified by CsCl gradient ultracentrifugation, titered using a cytopathic effect assay, and stored at −80°C in Hank’s balanced salt solution (HBSS).

Adenovirus-mediated gene delivery efficacy

The expression of eNOS and GFP and β-gal was determined first in vitro in cultured bovine pulmonary artery endothelial cells (CPAE; ATCC, Manassas, VA) by fluorescence microscopy of GFP and X-gal staining, respectively. The percentage of CPAE that expressed GFP or β-gal in vitro was assessed by fluorescence microscopy of GFP and X-gal staining, respectively. The percentage of CPAE that expressed GFP or β-gal was similar when cells were exposed to the same amount of adenovirus for 24 h.

The expression of eNOS and GFP in CPAE was also assessed by polymerase chain reaction (PCR) amplification and western immunoblotting as previously performed [21, 22]. For PCR amplifications, reverse transcribed RNA with the T-Primed First-Strand cDNA kit (Amersham Biosciences, Piscataway, NJ) was used in the presence of either (i) the bovine eNOS sense primer (5′-CACACTCGTGTTGGCGGTCGAG-3′) and anti-sense primer (5′-GGATTTTGGCCCATTTGCGGTCGAG-3′), which provided a 363-bp fragment; or (ii) the Aequorea victoria GFP sense primer (5′-AGAGTGGCATGCGGGCAAGGTT-3′) and anti-sense primer (5′-GCCATCGCCAATTTGAGGTATT-3′), which provided a 720-bp fragment or (iii) the housekeeping gene β-actin sense primer (5′-GACCTCTATCGCCACACAGT-3′) and anti-sense primer (5′-GTCTAGCTGACTCGTCTGCTG-3′), which provided a 221-bp fragment. The PCR parameters were as follows: 35 amplification cycles at 95°C for 30 s and 72°C for 2 min. For eNOS and GFP protein levels, the CPAE were homogenized in 200 mM Tris–HCl, 100 mM NaCl, 2 mM ethylenediaminetetraacetic acid (EDTA), 2 mM ethylene glycol tetraacetic acid (EGTA), 10% glycerol and 2 mM phenylmethylsulfonyl fluoride. Protein samples (25 μg) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto a polyvinylidene fluoride (PVDF) membrane. The membranes were soaked in 5% non-fat dry milk in 10 mM Tris-base, 250 mM NaCl and 0.2% Tween 20 (TBS-T) and incubated for 90 min in the presence of mouse eNOS (1:500) or a rabbit GFP (1:200) anti-serum (BD Biosciences, Palo Alto, CA). Then the membranes were washed and incubated for 45 min with a 1:2000 diluted anti-mouse or anti-rabbit horseradish peroxidase–conjugated IgG (Jackson Laboratories, West Grove, PA). The blots were washed and the immunoreactivity was visualized by immersion in the chemiluminescence solution (ECL; Amersham Biosciences) and exposed on X-OMAT film (Eastman Kodak, Rochester, NY).

The in vivo gene delivery was assessed in Sprague–Dawley rats after a single intravenous administration of the adenovirus that express eNOS and GFP (2 × 1010 p.f.u.) into the systemic circulation through the jugular vein using the procedure outlined below in renal failure rats. One week after adenovirus injection, a time point where gene expression has been reported to be maximal [23], the rats that received the vehicle or the adenovirus were anesthetized with pentobarbital (Somnotol, 50 mg/kg i.p.; MTC Pharmaceuticals, Cambridge, Ontario, Canada) and exsanguinated. The left kidney, the thoracic aorta, mesenteric arteries and the liver were harvested, and rinsed with and cleaned of blood. The tissues were immersed in OCT Embedding Medium (Tissue-Tek, Sakura Finetek, Torrance, CA), quickly frozen and stored at −80°C. Tissue sections (7 μm) were mounted on glass slides and used to assess eNOS and GFP expression by immunofluorescence analysis with confocal microscopy as previously performed [24, 25]. Tissue sections were overlayed with a 1:250 diluted primary mouse anti-eNOS antibody (BD Biosciences) followed by an Alexa Fluor 594 conjugated secondary anti-mouse antibody or an Alexa Fluor 488 conjugated rabbit anti-GFP antibody (Molecular Probes, Eugene, OR). The sections were then washed and counter stained by incubation with an 1:100 diluted Alexa Fluor 488 conjugated Phalloidine (Molecular Probes), which reveals actin filaments. Endothelial localization in blood vessels was confirmed using a rabbit anti-von Willebrand factor (Dako, Glostrup, Denmark), followed by incubation with an Alexa Fluor 594 conjugated rabbit anti-GFP antibody (Molecular Probes, Eugene, OR). The sections were then washed and mounted under controlled humidity and temperature conditions with a 1:1:12:1 light–dark cycle. Renal mass was reduced as previously described [26, 27] by ligating two or three branches of the left renal artery followed, 1 week later, by right nephrectomy under isoflurane anesthesia (1–2% C in Hank’s balanced salt solution (HBSS)). The vehicle or adenovirus prepara-

The animal experiments were approved by the Animal Care Committee of Laval University and were performed on 250 g male Sprague–Dawley rats (Charles River Laboratories, St-Constant, Quebec, Canada). The animals were allowed free access to standard laboratory rat chow and tap water and housed under controlled humidity and temperature conditions with a 12:12 h light–dark cycle. Renal mass was reduced as previously described [26, 27] by ligating two or three branches of the left renal artery followed, 1 week later, by right nephrectomy under isoflurane anesthesia (1–2% mixed with oxygen 100%). Sham-operated rats served as controls (n = 8). Two weeks after renal mass reduction, the rats were randomly divided into three groups and received an intravenous injection of the vehicle or the adenovirus that expresses eNOS or β-gal (n = 8 rats per group). The right jugular vein was approached by blunt dissection via a lateral neck incision. The vein was punctured with a 25 gauge winged needle attached to a 76 mm catheter filled with sterile HBSS. The vehicle or adenovirus prepara-

The experiments were performed as described above in the absence of anesthesia (n = 8). The effect of eNOS gene delivery on hypertension and renal failure and injury was assessed 4 weeks after adenovirus injection (corresponding to Week 6 of uremia). The dose of adenovirus and the timeframe of investigation was chosen based on a study by Chao’s group [28] reporting that kallikrein gene delivery in this rat remnant kidney model of chronic renal failure led to high level of expression in several tissues such as the heart, aorta, lung, kidney and liver and to a reduction in hypertension and renal failure for at least 4 weeks. Moreover, adenovirus-mediated eNOS gene delivery was also shown to produce long-term...
Results

Adenovirus-mediated gene delivery efficacy

In cultured CPAE cells exposed to the adenovirus, eNOS mRNA expression was very low in control cells and was significantly increased following adenovirus incubation (Figure 1A). Similarly, ENOS protein levels were detectable in control CPAE and were higher in cells exposed to the adenovirus (Figure 1B). As expected, GFP mRNA and protein levels were detectable exclusively in adenovirus-infected CPAE (Figure 1A and B, respectively). The difference in eNOS and GFP mRNA expression cannot be explained by differences in PCR reactions since β-actin mRNA amplification was similar in both control and adenovirus-infected CPAE.

The adenovirus-mediated gene delivery in vivo was confirmed by immunofluorescence analysis in normal rats 7 days after a single intravenous injection of either the adenovirus that contains eNOS and GFP or the vehicle. The expression of eNOS was modest in the endothelium of thoracic aorta from control rats that received the vehicle (Figure 1C). However, eNOS expression was 3.4 ± 0.2-fold greater in the thoracic aorta from animals that received the adenovirus and was also confined to the endothelium (Figure 1D). As expected, GFP expression was undetectable in the thoracic aorta from control rats but highly expressed in the endothelium exclusively in aorta (Figure 1F) and mesenteric arteries (data not shown) from animals that received the adenovirus. In the kidney, GFP expression was detected at low levels in glomeruli, but at higher levels in the wall of small renal arteries (Figure 1H) and was undetectable in the tubular cells. In the liver, GFP expression was mainly detected in Kupffer cells, localized at the edge of sinusoids and anchored to sub-endothelial structures (data not shown).

eNOS gene delivery in renal failure rats

Changes in systolic blood pressure. Prior to adenovirus administration, systolic blood pressure was significantly higher in renal failure rats as compared to sham-operated normal controls (150 ± 7 versus 120 ± 3 mmHg; P < 0.01, Figure 2). Thereafter, systolic blood pressures further increased over time in rats with reduced renal mass receiving the vehicle and the control adenovirus β-gal. In contrast, eNOS gene delivery prevented an increase in systolic blood pressure (Figure 2).

Changes in systolic blood pressure in renal failure rats that received the vehicle and the adenovirus β-gal were associated with cardiac hypertrophy. In fact, the heart weight to body weight ratio was significantly increased in rats with reduced renal mass receiving the vehicle and the adenovirus β-gal as compared to the normal controls (3.83 ± 0.12 versus 3.59 ± 0.12 versus 2.76 ± 0.08, P < 0.01) but was lower in those receiving the adenovirus that expressed eNOS (3.23 ± 0.11, P < 0.05).

Blood and renal parameters. Plasma and urinary NO2/NO3 concentrations were significantly lower in rats with reduced renal mass that received the vehicle and the control...
adenovirus β-gal as compared to the controls (Table 1). eNOS gene delivery, however, prevented the reduction in plasma and urine NO₂/NO₃ concentrations, which were similar or slightly higher to those in control animals.

Serum creatinine and proteinuria were increased in rats with reduced renal mass receiving the vehicle and the adenovirus β-gal as compared to the controls, whereas creatinine clearance was reduced (Table 2). eNOS gene delivery prevented the increase in proteinuria and attenuated the rise in serum creatinine and the reduction in creatinine clearance. A positive correlation was found between serum creatinine and systolic blood pressures in the different groups of rats ($r = 0.69$, $P < 0.001$) indicating a relationship between renal function and hypertension that were both improved by eNOS gene delivery.
As compared to the controls, ET-1 concentrations were higher in the renal cortex and urine of rats with reduced renal mass receiving the vehicle and the adenovirus β-gal but were lower in rats receiving the adenovirus that expresses eNOS (Table 2). Moreover, we found a positive correlation between ET-1 concentrations in the renal cortex or urine and serum creatinine values (\(r = 0.68\) and 0.82, respectively, \(P < 0.001\)) indicating a relationship between renal ET-1 production and the decline in renal function that were also improved by eNOS gene delivery.

Renal morphology. Renal injury in renal failure rats comprised marked blood vessel media hypertrophy and tubular atrophy, moderate focal and segmental glomerular sclerosis and diffuse interstitial fibrosis (Table 3 and Figure 3B). Similar morphological injury was observed in rats with reduced renal mass that received the control adenovirus β-gal (Figure 3C). However, eNOS gene delivery attenuated the renal vascular, glomerular and tubular injury (Table 3, Figure 3D). The renal protective effect of eNOS gene delivery was also associated with a reduction of collagen deposition in the tubulointerstitial space as compared to rats with reduced renal mass that received the vehicle and the control adenovirus β-gal (\(P < 0.05\), Figure 4).

Discussion

In the present study, we document that eNOS gene delivery in rats with reduced renal mass prevents the aggravation of hypertension and slows down the decline in renal function due, at least in part, to a marked reduction in renal vascular, glomerular and tubular injury. These protective effects after eNOS gene delivery are related to an improvement of NO release indicating that inappropriate eNOS/NO activity in chronic renal failure conditions plays a pivotal role in cardiovascular disease and renal failure progression.

A major finding in the present study is that eNOS gene delivery in rats with renal failure prevented the aggravation of hypertension that was observed in renal failure rats receiving the vehicle and the control adenovirus β-gal. Indeed, systolic blood pressure was similar in rats with reduced renal mass that received the adenovirus eNOS at Week 4 after gene delivery as compared to prior to adenovirus administration (Figure 2). This anti-hypertensive effect may well be related to the fact that eNOS gene delivery prevented a decrease in NO availability normally observed in rats with reduced renal mass. Plasma and urine NO\(_2/\text{NO}_3\) concentrations at Week 4 after gene delivery were higher in renal failure rats that received the adenovirus that expresses eNOS as compared to those that received the vehicle or the control adenovirus β-gal (\(P < 0.05\), Figure 4).
the large conduit (thoracic aorta) and resistance arteries (mesenteric) as revealed by immunofluorescence analysis. The endothelial-specific distribution is consistent with previous observations that adenovirus-mediated gene delivery in uninjured arteries in vivo is limited to the endothelium [23, 33, 34]. However, in tissues with fenestrated endothelium and basement membrane, gene delivery was detected in sub-endothelial cells such as Kupffer cells in the liver and the wall of small renal arteries as reported in this study. The possibility that eNOS expression improves the vascular endothelial function after systemic adenovirus administration is further supported by studies showing that eNOS gene delivery in vessels from Ang II-induced hypertensive rats restores endothelial NO release in the renal medulla may increase local blood flow and sodium and water excretion, thus attenuating volume expansion in renal failure animals [42]. In keeping with this, eNOS gene delivery prevented an increase in proteinuria even 4 weeks after adenovirus eNOS administration. The possible effect of eNOS on renal hemodynamics is consistent with studies documenting that the renal vasculature is highly sensitive to changes in NO release [43, 44]. NO is a potent vaso-dilator of both afferent and efferent arterioles [45] and, normally, counteracts the potent vasoconstrictor effect to Ang II and ET-1 [46, 47]. Moreover, the improvement of NO release in the renal medulla may increase local blood flow and sodium and water excretion, thus attenuating volume expansion in renal failure animals [48].

The renal vascular, glomerular and tubular protective effect of eNOS gene delivery in rats with reduced renal mass may also be related, in part, to the marked attenuation in renal ET-1 production as indicated by the reduction in renal cortex ET-1 levels and urinary ET-1 excretion. In fact, the latter has been previously reported to be positively correlated with the increase in serum creatinine and proteinuria and to be related to ET-1 overproduction in preglo-merular arteries and glomeruli [26, 27]. Endothelial NO release is a well-known mechanism limiting ET-1 production [40, 49]. Similar to eNOS gene delivery, supplementation with L-arginine in renal failure rats has been documented to reduce proteinuria together with the glomerular ET-1 overproduction [10]. In addition, NO has also been shown to inhibit the mitogenic response and the extracellular matrix deposition in mesangial cells induced by ET-1 as well as Ang II [50, 51].
Of particular interest, eNOS gene delivery in rats with reduced renal mass increased NO availability without supplementation with either L-arginine or BH$_4$ indicating that the substrate and cofactor concentrations were sufficient for appropriate eNOS activity. We cannot exclude the possibility, however, that the protective effect resulting from an early increase in NO availability after eNOS gene delivery might have contributed to the maintenance of L-arginine and BH$_4$ concentrations. In fact, early supplementation with L-arginine or BH$_4$ also improved NO release associated with cardiovascular and renal protection in renal failure rats [10, 12]. On the other hand, the renal protective effect of eNOS gene delivery in renal failure rats may attenuate the accumulation of endogenous inhibitors of eNOS such as ADMA [15] and the exaggerated production of superoxide anions [52]. In addition, the improvement of NO release may also reduce the effect of Ang II on superoxide formation through NADPH oxidase [53], which is consistent with the observation that eNOS gene delivery prevents superoxide formation in blood vessels from Ang II-induced hypertensive rats [35]. Although these protective effects remain to be clarified, our results suggest that early protection of the eNOS/NO activity is pivotal in slowing down the progression of chronic renal failure.

In conclusion, this study reveals that eNOS gene delivery in rats with reduced renal mass improves NO availability that may prevent the worsening of hypertension and reduce the rate of progression of renal failure and injury. Thus, improvement of NO availability should be considered in the management of hypertension and renal failure progression in chronic renal failure.

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References


\[\text{eNOS gene delivery in renal failure rats}\]


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