Vascular Gene Transfer of Phosphomimetic Endothelial Nitric Oxide Synthase (S1177D) Using Ultrasound-Enhanced Destruction of Plasmid-Loaded Microbubbles Improves Vasoreactivity

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Background—Local gene therapy has enormous potential for the treatment of vascular disease. We determined whether diagnostic ultrasound-mediated destruction of plasmid-loaded albumin microbubbles is a feasible and efficient technique for local vascular gene delivery. For gene transfer, we used a phosphomimetic, active endothelial nitric oxide synthase (eNOS) construct in which Ser1177 was replaced by aspartic acid (S1177D) and exhibits a 2-fold higher basal activity than the wild-type enzyme.

Methods and Results—Gas-filled microbubbles (3.0 ± 1.2 μm) were created by sonication of 5% human albumin in the presence of plasmid DNA encoding for LacZ or eNOS S1177D. Porcine coronary arteries were perfused with DNA-loaded albumin microbubbles in vitro, exposed to diagnostic ultrasound (5 seconds), and incubated for a further 24 hours. Detection of the β-galactosidase in LacZ-transfected vessels revealed a predominant staining of endothelial cells without any functional impairment of vasoreactivity. Western blotting demonstrated the expression of the eNOS S1177D construct in extracts from the transfected segments. Vascular responsiveness was tested with prostaglandin F2α and the NOS inhibitor N-nitro-L-arginine. Compared with segments treated with the expression plasmid alone, the contractile response to prostaglandin F2α was impaired in segments transfected with eNOS S1177D, whereas the contractile response to the administration of N-nitro-L-arginine was markedly enhanced.

Conclusions—Ultrasound-mediated destruction of eNOS S1177D DNA-loaded albumin microbubbles is a feasible and efficient method for vascular gene transfection. Transfection resulted in significant protein expression and enhanced NO-mediated relaxation of bradykinin-stimulated porcine coronary arteries. (Circulation. 2002;105:1104-1109.)

Key Words: endothelium ■ echocardiography ■ gene therapy ■ nitric oxide synthase

Although gene therapy holds great promise as a treatment for disease, current clinical application is hampered by the development of safe and efficient systems for local gene delivery to a specific tissue or organ. Two categories of delivery vehicles (“vectors”) are currently available. Nonviral delivery can be performed by the direct application or injection of DNA or mixing the DNA with agents that facilitate the passage of the gene of interest across the cell membrane. Such approaches are generally associated with a low transfection efficiency and transient expression of the gene product. As a result, viral vectors are the tools of choice in most of the current gene therapy approaches. The use of viruses significantly increases the efficacy of transfection because of the specific viral machinery that has specifically evolved to introduce foreign DNA into mammalian cells. However, attempts to transport genes into cells through the use of viral vectors have been hampered by the fact that viral proteins elicit an immune response within the targeted host/tissue. Specifically, adenovirus-facilitated transfection of the endothelium has been shown to induce profound inflammatory activation of the endothelial lining, an effect that very probably counteracts any beneficial effects of the gene transfection procedure.

Recently, ultrasound-induced microbubble destruction has been proposed as a new technique for local delivery of drugs and genes to specific target tissues, including the heart. This technique uses albumin bubbles to encase an expression vector until the site of transfection is reached; thereafter, an ultrasound probe is used to burst the bubbles, thus distributing material in a specific area of interest. The microbubble approach has been previously used to deliver colloidal particles to tissues after microvessel rupture.

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transgene by ultrasound has been shown to significantly increase myocardial gene expression in rats as well as to enhance cationic lipid-mediated gene transfer into primary tumors. However, it is unclear whether or not ultrasound can be used to facilitate transfection with pure plasmid DNA. More importantly, since a normal endothelial function is thought to be essential for the prevention of atherosclerotic lesion development and smooth muscle cell proliferation, the cardiovascular application of ultrasound-induced gene transfection should not be associated with the functional damage to the endothelial lining described in initial experiments. The aim of the present investigation, therefore, was to investigate whether or not ultrasound-induced destruction of plasmid-loaded microspheres results in efficient transfer of a gene to the vascular wall of coronary arteries without impairing the functional activity of the endothelial cell layer. Because nitric oxide (NO) is a crucial mediator of the atheroprotective effects of the functionally intact endothelium, we used a phosphomimetic endothelial nitric oxide synthase (eNOS) construct, which simulates activation of the eNOS by phosphorylation of the amino acid Ser 1177.

Mimicking phosphorylation of eNOS by replacing Ser 1177 with aspartate enhances the basal activity of the enzyme without the need for an increase in the intracellular concentration of calcium.

Methods

Plasmids

For transfection of the vessels, pcDNA3.1 plasmids (Invitrogen) were used, which express the gene of interest under a cytomegalovirus promoter. The transfection efficiency was determined by use of a plasmid encoding the β-galactosidase (pcDNA3.1-LacZ) compared with an empty vector control (pcDNA3.1). Phosphomimetic eNOS was cloned in the pcDNA3.1- \textit{myc-his}-vector and mutated as previously described.

Preparation of DNA-Loaded Microbubbles

Gas-filled albumin microbubbles were created by slight modification to the method described previously by Reisner et al. Briefly, 2 mL of a 5% solution of human albumin (BSD BW) containing 20 μg of DNA (propidium iodide–bound DNA, pcDNA-LacZ, pcDNA 3.1, or pcDNA-eNOS S1177D) was drawn into a 5-mL syringe, which was connected by a 3-way tap to another 5-mL syringe containing 2 mL of gas (room air or perfluorocarbon). The DNA-albumin solution was sonicated with a sterile (20 kHz) sonication horn (Sonifier Desintegrator model W-450, Branson Ultrasonic S.A.), which was introduced into the syringe. Continuous sonication (output setting 7) was performed for 20 seconds. Then, 2 mL of gas was introduced into the sonicating chamber over a period of 10 seconds. During the sonication process, the temperature increased to 55°C. After sonication, the upper foamy layer and lower clear layer were mixed and 1 mL of this solution was diluted with 4 mL of nonsonicated albumin. The incorporation of DNA into the albumin coating of the microbubbles was confirmed by fluorescence microscopy with the use of purified propidium iodide–labeled DNA. The microbubble size (3.0±1.2 μm) was determined by computer-assisted microscopic analysis (KS 300 Imaging system 3.0, Carl Zeiss Vision). The microbubble concentration (2.1±0.5×10^10 bubbles/mL) was measured by cell counter (Technicon H-3 RTX, Bayer).

Preparation of Porcine Coronary Artery

Pig hearts were obtained from a local slaughterhouse, placed immediately into ice-cold physiological salt solution, and transported to the laboratory. Porcine coronary arteries (PCAs) were dissected and cleaned of adventitial adipose and connective tissue. Vessel segments (~40 mm in length; mean external diameter, 2.4 to 2.8 mm) were excised and the side branches sealed with surgical clips. Segments were cannulated at both ends, placed in vessel chambers, and rinsed with 2 mL of DMEM and 2 mL of PBS. The solutions did not recirculate. Immediately thereafter, vessel segments were perfused with DNA-loaded microbubbles and exposed to ultrasound, rinsed with 2 mL of PBS and 2 mL of DMEM, and perfused (5 mL/h, 37°C) with DMEM containing 2% fetal calf serum for 24 hours. At the end of the incubation period, each segment was cut into rings for histochemical, biochemical, and functional analysis.

Microbubble Disruption

PCAs were mounted in vessel chambers connected to an infusion pump and perfused with the DNA-microbubble solution. Diagnostic ultrasound was applied by means of a commercially available system (System Five, GE Vingmed) with a transducer working in harmonic mode (2.2 to 4.4 MHz). Total image depth was 8 cm, and the coronary arteries were positioned horizontally at the focus point at 5 cm (size of sector, 3.6×2.7 cm). Continuous, high-energy ultrasound (mechanical index, 1.2; frame rate, 172.9 frames per second) was applied (5 or 30 seconds) to vessels perfused (from 2 to 10 mL/minute) with DNA-loaded microbubbles.

Histochemical Staining for LacZ

To determine whether the transfection protocol resulted in the expression of recombinant protein, histochemical staining for LacZ expression was performed. PCA segments were fixed for 15 minutes in 1:25% glutaraldehyde (Sigma Chemical) before being stained with 5-bromo-4-chloro-3-indolyl-D-galactopyranoside (X-Gal reagent; ICN). To assess the localization of LacZ expression in the vessel wall, stained vessels were embedded in paraffin and cut into thin sections.

Protein Isolation and Western Blotting

For isolation of whole protein extracts, PCAs were homogenized in liquid nitrogen and lysed in a buffer containing 10 mMol/L Tris-HCl, pH 8, 1% Triton-X-100, 5 mMol/L EDTA, pH 8, 0.32 mol/L sucrose, 2 mMol/L DTT, and 1 mMol/L PMSF (30 minutes, 4°C). After centrifuging at 15 000 rpm for 15 minutes at 4°C, protein content was determined by Bradford assay (Bio-Rad), with bovine serum albumin used as standard.

Proteins (35 μg) were separated by SDS-PAGE electrophoresis (8%) and were blotted onto PVDF membranes (Millipore). Nonspecific binding was blocked (5% nonfat dry milk powder in TBS/Tween [50 mMol/L Tris-HCl, pH 8, 150 mMol/L NaCl, 2.5 mMol/L KCl, and 0.1% Tween 20]). After incubation with primary antibodies (c-myc [1:250 in 5% milk, 2 hours] or eNOS [1:1000 in 5% milk, 2 hours]; Santa Cruz Biotechnology), blots were washed 3 times in TBS/Tween and then incubated with horseradish peroxidase–conjugated secondary antibody for 1 hour (1:4000). Enhanced chemiluminescence was performed according to the instructions of the manufacturer (Amersham Pharmacia Biotech).

β-Galactosidase Assay

pcDNA-LacZ–transfected cell lysate (15 μL) was mixed with cleavage buffer containing β-mercaptoethanol and ortho-nitrophenyl-β-D-galactopyranoside (β-Gal Assay Kit, Invitrogen). After incubation at 37°C for 30 minutes, the absorbance was photometrically measured at 420 nm. A pcDNA-3.1(−) transfected cell lysate was used as control.

LDH Activity and Trypan Blue Staining

Lactic dehydrogenase (LDH) activity was measured in the vessel perfusate by a commercially available assay (Boehringer Mannheim). Vessels were stained with trypan blue (1:100 in PBS) to detect endothelial cell injury.
Ultrasound-Enhanced Gene Transfer of β-Galactosidase

PCA segments were perfused with pcDNA-LacZ–loaded microbubbles and exposed to ultrasound. After a further incubation period of 18 to 20 hours, intense blue staining, indicating expression of LacZ protein, was detected in the region of the segment targeted by ultrasound (Figure 1A). Segments perfused with microbubbles carrying an empty vector but otherwise treated identically showed no blue staining. Interestingly, the staining was most intense at that end of the vessel segment, where the microbubbles entered the ultrasound sector, that is, the area exposed to the highest concentration of microbubbles, and gradually decreased in the direction of flow (Figure 1A). β-Galactosidase was expressed in endothelial cells, and histochemical analysis revealed that >90% of the endothelial cells were positively stained, whereas only sporadic staining of subintimal smooth muscles cells was detected (Figure 1B).

The function of the β-galactosidase introduced into the arteries was quantified by measuring the enzymatic activity. β-Galactosidase activity was detectable in arteries perfused with LacZ-plasmid, but in the absence of microbubbles (Figure 1C), a similar signal was observed in homogenates prepared from segments perfused with the LacZ-loaded microbubbles but not exposed to ultrasound. The combination of LacZ-loaded microbubbles and ultrasound treatment, however, markedly increased the β-galactosidase activity measurable in PCA homogenates (Figure 1C). Increasing the flow rate of the microbubble perfusate affected the efficiency of gene transfer. Whereas a flow rate of 2 mL/min slightly increased transfection of the vessels (1.5±0.2-fold compared with static conditions), a further increase of the flow rate to 10 mL/min resulted in a reduction of β-galactosidase activity (Figure 2A through C). The reduction of transfection efficiency at 10 mL/min was completely compensated by increasing the ultrasound exposure time from 5 to 30 seconds (Figure 2C). The expression of β-galactosidase was concentration-dependent and greatest at the highest dose tested (Figure 2D). Similar data were obtained when perfluorocarbon instead of room air was used for preparation of the microbubbles (data not shown).

Ultrasound-Enhanced Gene Expression Does Not Impair Endothelial Function

Because previous studies demonstrated that ultrasound can induce endothelial cell injury, we determined the effect of LacZ transfection on the bradykinin-induced endothelium-
dependent relaxation of rings cut from the transfected vessels. The bradykinin-induced relaxation of U46619-precontracted PCA rings was similar in untreated control vessels and vessels exposed to microbubbles and ultrasound ($\text{pD}_2 [-\log \text{EC}_{50}]$ values being 9.28±0.07 and 9.87±1.35 in control and treated groups, respectively, $n=4$, $P=0.6778$; Figure 3). Moreover, ultrasound exposure did not induce any histologically detectable alteration of the vessel wall (see Figures 1B and 2B). Furthermore, trypan blue staining of the endothelium and measurement of LDH activity in the perfusate excluded a cytotoxic effect of the treatment. Although perfusion with DNA-loaded microbubbles (10 μg/mL, 2 mL/min) and ultrasound exposure (5 seconds) did not affect LDH levels (88.8±16%), a 6.3-fold increase in LDH level was observed after treatment with the cytotoxic substance doxorubicin (5 μg/mL), which was used as positive control.

Transfection of eNOS S1177D Enhances NO-Mediated Relaxation

To determine whether or not the ultrasound-enhanced destruction of microbubbles carrying a gene construct leads to alterations in coronary artery function, PCAs were transfected with the eNOS S1177D-construct. Western blot analysis of the area of the segments targeted by the ultrasound probe revealed a pronounced increase in the expression of eNOS protein (Figure 4A). However, the differential expression between the two groups was best seen after blotting for myc because the S1177D construct contained a myc-tag (Figure 4B). To assess whether or not the introduction of S1177D into endothelial cells was associated with an increase in NO production, we assessed the contractile response to the application of L-NA in vessels contracted with prostaglandin $\text{F}_2\alpha$ ($\text{PGF}_{2\alpha}$). Compared with the control group, $\text{PGF}_{2\alpha}$-induced contraction was significantly impaired in PCA rings

Figure 2. Influence of flow and microbubble concentration on effectiveness of gene transfer. A and B, Vessels were exposed to pcDNA3.1-LacZ (0.1 or 2 μg/mL) either under static conditions or during continuous perfusion (2 mL/min) as indicated, and β-galactosidase expression was assessed ($n=3$ to 11). En face histochemical staining is presented in A; cross sections of the same vessels are shown in B. C, Effect of flow rate on gene expression: β-galactosidase activity in vessel segments exposed to pcDNA3.1-LacZ–loaded albumin microbubbles (DNA concentration, 2 μg/mL) and treated with ultrasound (5 or 30 seconds) and either perfused at a rate of 2 or 10 mL/minute. Data represent mean±SEM; $^*P<0.05$. D, Relation between plasmid concentration applied and resulting expression of β-galactosidase (flow rate, 2 mL/minute; 5 seconds of ultrasound exposure). Data are mean±SEM, $^*P<0.05$ ($n=3$ to 6).

Figure 3. Effect of ultrasound-induced disruption of albumin-coated microbubbles on endothelium-dependent NO-mediated relaxation. Concentration-relaxation curves show the effect of the cumulative application of bradykinin (30 pmol/L to 0.3 μmol/L) to rings of PCA precontracted with U46619. Rings were prepared from segments of coronary artery exposed to DNA-coated microbubbles (2 μg/mL plasmid concentration; flow rate, 2 mL/min) in the presence or absence of ultrasound (5 seconds). Data are expressed as mean±SEM, with segments from 4 different animals used.
prepared from segments transfected with eNOS S1177D (Figure 4C). In addition, there was an ≈2-fold increase in the L-NA–induced contraction in eNOS S1177D–transfected vessel segments compared with vector-transfected vessels (Figure 4D). These results indicate that the ultrasound-enhanced delivery of the eNOS S1177D construct to PCAs significantly enhanced vascular NO production.

Discussion
The results of this study demonstrate that ultrasound-mediated destruction of albumin-coated microbubbles loaded with naked plasmid-DNA is a feasible and efficient technique for local gene delivery within the vascular wall. Indeed, the application of ultrasound to vessels perfused with albumin microbubbles carrying plasmids encoding either β-galactosidase or eNOS resulted in level of endothelial cell transfection significantly greater than that observed in vessels exposed to the DNA-loaded microbubbles in the absence of ultrasound. Moreover, the transfection of endothelial cells with the eNOS S1177D construct was associated with enhanced NO-mediated responses, indicating an increased NO synthesis within transfected vessel segments.

Progress in local cardiovascular gene therapy has been hampered by problems relating to the safety and practicality of using viral vectors and the inefficiency of current nonviral transfection techniques. In this study, we were able to demonstrate that naked pcDNA, diluted in 5% human albumin and sonicated in the presence of air or perfluorocarbon gas, can be incorporated directly into the microbubble shell. No loss in the activity of the plasmid DNA was associated with the sonication process or ultrasound-induced bubble destruction in the vessel, as reported previously. Previous investigators have successfully used ultrasound to deliver labeled red blood cells, polymer microspheres, vascular endothelial growth factor, or oligonucleotides to different target tissues. Likewise, enhancement of gene transfection by ultrasound has been reported previously. Although the latter studies focused on the tissue-specific and organ-targeted delivery of genes, the application of the technique was associated with the disruption of microvascular endothelial cells. Our study is the first to document efficient gene transfer to the endothelial cells of conductance arteries without traumatizing the vascular wall by using infusion balloons or infiltration devices. In fact, despite efficient transgene expression, the functional integrity of the transfected endothelial cell layer was well preserved after ultrasound treatment. Thus, ultrasound-mediated destruction of albumin-coated microbubbles loaded with naked plasmid DNA appears to be ideally suited for the delivery of a gene to the endothelial cell layer of conductance vessels prone to atherosclerotic lesion development and/or progression.

Exactly why transfection of the endothelial lining with naked DNA derived from albumin coated air bubbles is so efficient remains to be determined. Previous studies have shown that ultrasound activation of gas-filled microbubbles with acoustic power of 0.8 to 1 W/cm² is associated with reversible membrane damage and endothelial injury, whereas no structural alterations were observed with an acoustic power of 0.6 W/cm². Clinically used ultrasound machines similar to the one used in the present study are capable of delivering an acoustic output up to 0.44 W/cm². In line with the lack of any functional impairment, we did not observe any structural alterations of the endothelial lining after ultrasound treatment. Moreover, assessment of endothelial cell viability by trypan blue staining and measurement of LDH in the perfusate excluded a cytotoxic effect of the ultrasound-mediated destruction of the DNA-loaded albumin bubbles to the endothelium. Endothelium-derived NO exerts pivotal antiatherosclerotic effects. Moreover, the well-established vasculoprotective effects of regular physiological exercise have been attributed to the shear stress–induced stimulation of NO release. In
fact, shear stress results in the activation of protein kinase B (Akt), which phosphorylates eNOS on Ser1177 to enhance enzyme activity 2- to 3-fold over basal levels without the need for an increase in intracellular Ca^{2+}.\textsuperscript{11} We therefore used an eNOS construct for our transfection studies in which Ser1177 was replaced by alanine to mimic the physiological activation of eNOS. Indeed, efficient transfection of eNOS Ser1177D attenuated PGF_{2\alpha}-mediated precontraction by $\approx 60\%$ and increased the constriction observed after the application of L-Na, a response we have previously reported to reflect the inhibition of NO production by eNOS in response to isometric contraction.\textsuperscript{14}

Taken together, the results of present study demonstrate that ultrasound-induced destruction of albumin microbubbles coated with a naked plasmid-DNA is an efficient technique for local gene delivery to the vascular wall and does not impair the functional integrity of the endothelium.

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