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Nitric Oxide-Loaded Echogenic Liposomes for Nitric Oxide Delivery and Inhibition of Intimal Hyperplasia

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Objectives We sought to develop a new bioactive gas-delivery method by the use of echogenic liposomes (ELIP) as the gas carrier.

Background Nitric oxide (NO) is a bioactive gas with potent therapeutic effects. The bioavailability of NO by systemic delivery is low with potential systemic effects.

Methods Liposomes containing phospholipids and cholesterol were prepared by the use of a new method, freezing under pressure. The encapsulation and release profile of NO from NO-containing ELIP (NO-ELIP) or a mixture of NO/argon (NO/Ar-ELIP) was studied. The uptake of NO from NO-ELIP by cultured vascular smooth muscle cells (VSMCs) both in the absence and presence of hemoglobin was determined. The effect of NO-ELIP delivery to attenuate intimal hyperplasia in a balloon-injured artery was determined.

Results Coencapsulation of NO with Ar enabled us to adjust the amount of encapsulated NO. A total of 10 μl of gas can be encapsulated into 1 mg of liposomes. The release profile of NO from NO-ELIP demonstrated an initial rapid release followed by a slower release during the course of 8 h. Sixty-eight percent of cells remained viable when incubated with 80 μg/ml of NO/Ar-ELIP for 4 h. The delivery agent of NO to VSMCs by the use of NO/Ar-ELIP was 7-fold greater than unencapsulated NO. We discovered that NO/Ar-ELIP remained an effective delivery agent of NO to VSMCs even in the presence of hemoglobin. Local NO-ELIP administration to balloon-injured carotid arteries attenuated the development of intimal hyperplasia and reduced arterial wall thickening by 41 $\pm$ 9%.

Conclusions Liposomes can protect and deliver a bioactive gas to target tissues with the potential for both visualization of gas delivery and controlled therapeutic gas release. (J Am Coll Cardiol 2009;54:652–9) © 2009 by the American College of Cardiology Foundation

Nitric oxide (NO) is a potent bioactive gas with vasodilatory, anti-inflammatory, antithrombotic, antiproliferative, and possibly antiatherogenic properties (1,2). The desirable characteristics of NO in modulating the development of various vascular diseases have prompted the development of various NO precursors, synthetic NO promoters such as L-arginine, endothelial NO synthase gene, NO donors, and NO gas. One of the most popular methodologies for NO gas delivery clinically is via inhalation (3–5). In 1999, the U.S. Food and Drug Administration approved an inhaled formulation of NO gas for the treatment of patients with persistent pulmonary arterial hypertension (6). Another potential use of NO would be to modulate neointimal hyperplasia (7). Although the delivery of NO to the arterial wall has a number of potential benefits, successful NO delivery to targeted tissues is challenging because of the presence of endogenous NO scavengers such as hemoglobin (5).

Our laboratory has developed a number of liposomal formulations for molecular imaging and drug and gene delivery (8–12). The encapsulation of air into these liposomal formulations results in a contrast agent that is suitable for ultrasound image enhancement and is stable in serum at 37°C for a prolonged duration (9). In this study, we demonstrated that gases such as NO can also be encapsulated into our echogenic liposomes (ELIP), resulting in a "theranostic" agent with both echogenic and bioactive gas-delivery properties.

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The specific aims of this study were to: 1) encapsulate NO into ELIP; 2) adjust the encapsulated gaseous composition by the inclusion of argon (Ar) to modulate the rate of NO release; 3) determine the protective effects of NO encapsulation into ELIP for delivery to vascular smooth muscle cells (VSMCs) in vitro in the presence of hemoglobin, an endogenous NO scavenger; and 4) demonstrate the potential therapeutic effects of NO delivery from NO/Ar-ELIP in attenuating neointimal hyperplasia in injured common carotid arteries of cholesterol-fed rabbits.

Methods

Preparation of NO-containing liposomes (NO-ELIP) and NO/Ar-containing liposomes (NO/Ar-ELIP). We used liposomes composed of 1,2-dipalmitoyl-*sn*-glycero-3-ethylphosphocholine (Genzyme Corporation, Cambridge, Massachusetts); 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (Avanti Polar Lipids, Alabaster, Alabama); and cholesterol (Avanti Polar Lipids) at a mole ratio of 60:30:10. Liposomes were made by the previously developed pressured-freeze method with modification (13). In brief, after drying and hydrating lipid film, the liposomes were transferred to a 2-ml glass vial with a cap that was sealed with a Teflon-rubber septum. Then, NO (specialty gases of America, Inc., Toledo, Ohio) was washed and deoxygenated with a saturated NaOH solution. Ten milliliters of NO alone or a gaseous mixture of NO and Ar was injected into the glass vial through the Teflon-rubber septum by the use of a 12-ml syringe attached to a 27-G, 0.5-inch needle. The pressurized liposomal dispersion was frozen at −70°C with dry ice for at least one-half hour. The pressure in the vial was released by the removal of the cap, and the liposomal dispersion was allowed to thaw. Gas encapsulation, release profiles, and delivery characteristics of NO-ELIP or NO/Ar-ELIP were studied after thawing. The volume of encapsulated gas in liposomes was measured by the use of a syringe method as previously described (12).

The NO release study. The release of NO from pure NO-ELIP and NO/Ar-ELIP was determined by serial dialysis under sink conditions. A dispersion of 20 μl of NO-ELIP and 180 μl of phosphate-buffered saline (PBS) (donor buffer) was placed inside a dialysis tube (MWCO:12-14,000, Spectrum Laboratories, Inc., Rancho Dominguez, California) and dialyzed against 1 ml of PBS (receiving buffer). The dialysis tube was transferred to a fresh PBS solution and the release of NO was measured at 5, 10, 20, 30, 60, 120, 240, and 480 min. The measurement of NO release from NO-ELIP into the receiving buffer was based on the reaction between NO and oxygen to yield nitrite ($4NO + O_2 + 2H_2O \rightarrow 4NO_2^- + 4H^+$) (14). The nitrite concentration in the receiving solution was measured with a colorimetric NO assay kit (BioVision Inc., Mountain View, California). Because NO can exist in 2 different compartments in the dialysis system, namely encapsulated NO inside NO-ELIP or dissolved NO in the surrounding solution, NO-saturated mannitol solution was used as a control to correct for NO release from NO-ELIP. Thus, the nitrite concentration in the receiving buffer with the NO-ELIP or NO/Ar-ELIP subtracted by the nitrite concentration in the receiving solution with the mannitol was taken to be equivalent to the NO released from NO-ELIP or NO/Ar-ELIP at each time point.

Cell viability in the presence of NO/Ar-ELIP. Rat VSMCs were grown in 75-cm^2 flasks containing a medium of Dulbecco modified Eagle medium (DMEM) (GIBCO-BRL, Grand Island, New York) and 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 mg/ml streptomycin at 37°C under 5% CO_2 and 95% air. Cells obtained between passages 5 and 8 were used.

Abbreviations and Acronyms

- **Ar** = argon
- **CH** = cholesterol
- **DAF-2DA** = diaminofluorescein-2 diacetate
- **DMEM** = Dulbecco's modified Eagle medium
- **ELIP** = echogenic liposomes
- **FBS** = fetal bovine serum
- **NO** = nitric oxide
- **NO/Ar-ELIP** = nitric oxide/argon-containing liposomes
- **NO-ELIP** = nitric oxide-containing liposomes
- **PBS** = phosphate-buffered saline
- **VSMC** = vascular smooth muscle cell

Twenty-four hours before the experiment, rat VSMCs were seeded in 48-well plates in DMEM and 10% FBS. The medium was removed and replaced with a solution containing DMEM and 0.3% FBS. The concentration of FBS was decreased to minimize the interference with the NO assay. Differing concentrations of NO/Ar-ELIP were added to VSMC and incubated at 37°C for 4 h. After incubation, the medium was removed, and the cells were washed twice with PBS. Cell viability was measured with a commercially available calcein AM kit (Calbiochem, Gibbstown, New Jersey).

Delivery of NO into VSMCs. Thirty microliters of NO/Ar (1:9)-ELIP or NO/Ar saturated mannitol solution were incubated with cultured VSMCs in a 48-well plate for 5 min. The solutions were aspirated and VSMC were washed 3 times with 500 μl of PBS solution. The uptake of NO by VSMC was determined by measuring the nitrite concentration in cultured cells by use of the NO assay kit. In brief, in this reaction, nitrate reductase in the VSMC converts nitrate to nitrite. Nitrite then reacts with the chromogenic substrate, sulfanilamide, and N-(1-naphthyl) ethylenediamine to produce a color reaction that was determined according to the optical density at 540 nm (OD540) with a Tecan microplate reader (Tecan US Inc., Raleigh, North Carolina).

Delivery of NO in the presence of hemoglobin. The uptake of NO by VSMCs in the presence and absence of hemoglobin was measured by the NO-fluorescent probe diaminofluorescein-2 diacetate (DAF-2DA, Calbiochem) to localize NO production in cells by fluorescence microscopy. The probe DAF-2DA is a nonfluorescent agent that can readily diffuse into cells and be converted into diaminofluorescein-2 by cytosolic esterase inside living cells.

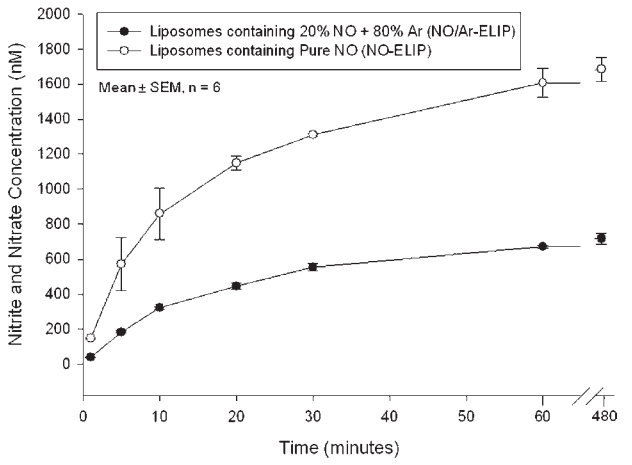

Figure 1 NO Release Profile

Illustrated is the amount of released NO when pure NO **(open circles)** or a mixture of 20% NO with 80% Ar **(closed circles)** were encapsulated into the liposomes. NO = nitric oxide; NO/Ar-ELIP = nitric oxide/argon-containing liposomes; NO-ELIP = nitric oxide-containing liposomes.

When NO is delivered into VSMC, NO reacts with diaminofluorescein-2, a nonfluorescent dye, to generate diaminofluorescein-2 triazole, a fluorescent product. In the presence or absence of 1 mg/ml of hemoglobin, VSMCs were incubated with 30 μl of NO/Ar-ELIP or NO saturated mannitol solution for 5 min. The incubating solution was removed, and VSMCs were washed 3 times with PBS. Ten microliters of DAF-2DA working solution (1:500 dilutions in PBS) were added to VSMC and incubated for 30 min in the dark. The DAF-2DA was removed and the VSMCs were washed 3 times with PBS. The uptake of NO by VSMCs under different conditions was qualitatively assessed by fluorescence microscopy at wavelength of 495 nm.

Balloon injury and local delivery of NO/Ar-ELIP in vivo. All animal experiments were approved by the Animal Welfare Committee at the University of Texas Health Science Center at Houston. Twenty-one male New Zealand White rabbits weighing 3.0 to 4.5 kg were used. The animals were fed an atherogenic diet, containing 0.2% cholesterol and 4% coconut oil for 2 weeks before balloon denudation. On the day of surgery, the rabbit was anesthetized with ketamine (35 mg/kg), xylazine (5 mg/kg), and 1% to 3% isoflurane. Balloon injury to the common carotid artery was performed using a 2-F Fogarty catheter (Edwards Lifesciences, Irvine, California).

A sheath was inserted into the right external carotid artery, and the catheter was introduced through the sheath into the common carotid artery. The balloon was inflated with 200 μl of saline solution and passed back and forth for 3 times in the common carotid artery over a distance of 3 cm. Immediately after balloon injury, liposomes containing Ar alone or a gaseous mixture of 10% NO and 90% Ar was injected locally into the distally occluded common carotid artery through the sheath and allowed to dwell for 2 min. The occluding suture was then released, the sheath removed, the external carotid artery ligated, the wound closed, and the rabbit allowed to recover. Carotid arteries subjected to a sham procedure, balloon-injured carotid arteries without treatment, and contralateral common carotid arteries without injury or treatment were used as controls.

The animals continued on a high cholesterol diet and were sacrificed 2 weeks after surgery. The carotid arteries were removed, cut into 2.5-mm segments, and fixed with PBS containing 4% formalin for 24 h. The fixed tissues were embedded in paraffin and sections were stained with hematoxylin and eosin for histologic examination. All sections from the injured segment of each artery were examined in a blind fashion using Image-Pro Plus (Media Cybernetics, Bethesda, Maryland). Measurements were made of the cross-sectional area of the lumen and of the areas enclosed by the internal and external elastic laminae. The intimal cross-sectional area of carotid artery segments was determined by subtracting the area of the lumen from the area enclosed by the internal elastic lamina. The medial area was determined by subtracting the area enclosed by the internal elastic lamina from the area enclosed by the external elastic lamina.

Statistical analysis. Statistical analysis between groups was performed by a *t* test or one-way analysis of variance with the use of SigmaStat (version 3.5, Systat Software Inc., Point Richmond, California) and Statistica (version 8.0, StatSoft, Inc., Tulsa, Oklahoma) software. Differences between multiple groups were assessed with the post-hoc Tukey HSD test for unequal N. A value of $p < 0.05$ was considered significant. All data are presented as mean ± SEM.

Results

Encapsulation and release of NO. Ten microliters of NO gas were encapsulated into 1 mg of liposomes by use of the

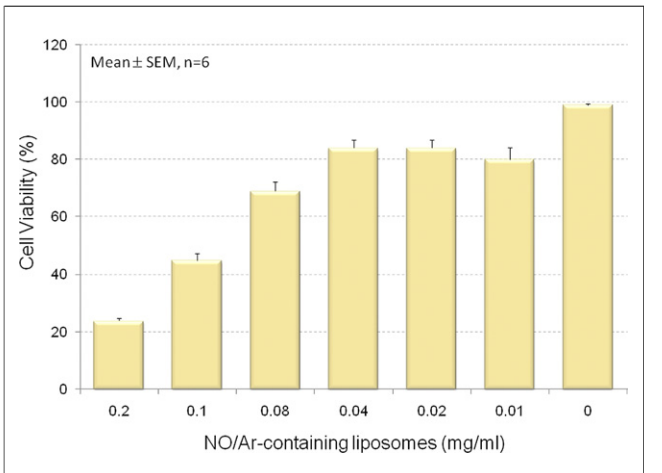

Figure 2 Cell Viability With NO-ELIP

Vascular smooth muscle cell viability at 4 h after differing amounts of NO/Ar (1:9)-containing liposomes. Viability was measured with the use of calcein AM. Abbreviations as in Figure 1.

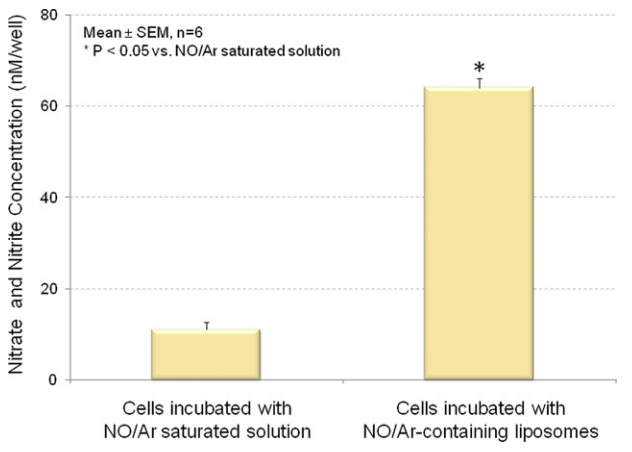

Figure 3 **NO Delivery Into Cultured Vascular Smooth Muscle Cells**

Cells were cultured in 48-well plates and incubated with NO/Ar (1:9)-saturated mannitol **(left)** or 30 μg of NO/Ar (1:9)-containing liposomes **(right)** for 10 min. The uptake of NO was expressed as nitrate and nitrite concentrations. Abbreviations as in Figure 1.

freezing under pressure method. The release of NO from NO-ELIP was rapid, with 50% of the encapsulated gas released within 10 min, followed by slower release over 8 h (Fig. 1). In contrast, NO release was slower in the presence of Ar (liposomes containing 20% NO and 80% Ar). The total volume of releasable NO was 2 μl from 1 mg 20% NO/80% Ar-containing liposomes, 1 μl from 1 mg 10% NO/90% Ar-containing liposomes, and 10 μl from 1 mg NO-ELIP. For the rest of our experiments, we used a NO/Ar ratio of 1 to 9, with a NO concentration of 0.045 μmol/mg lipids.

Cell viability in the presence of NO-ELIP. The effect of the lipid and gaseous components of 10% NO and 90% Ar on cell viability was evaluated by measuring the extent of calcein AM uptake in cultured VSMC. As shown in Figure 2, 68% of cells remained viable after incubating with 0.08 mg/ml NO/Ar-ELIP (2 μmol/l NO) for 4 h. Eighty-two percent of the cells were viable in a culture media containing 0.01 to 0.04 mg/ml NO-ELIP. We arbitrarily selected concentrations in the range from 0.01 to 0.04 mg/ml (containing 0.26 to 1.0 μM/l of NO) of NO/Ar-ELIP in subsequent in vitro cell culture experiments because of the relatively high cell viability and the substantial amount of NO that was deliverable by NO/Ar-ELIP.

Delivery of NO into cultured VSMCs. The uptake of NO by cells was measured on the basis of the corresponding nitrate and nitrite concentration by use of a colorimetric NO assay kit. The VSMCs were incubated with a solution containing 30 μg/ml NO/Ar-saturated mannitol or 30 μg/ml NO/Ar-ELIP. The amount of NO that can be

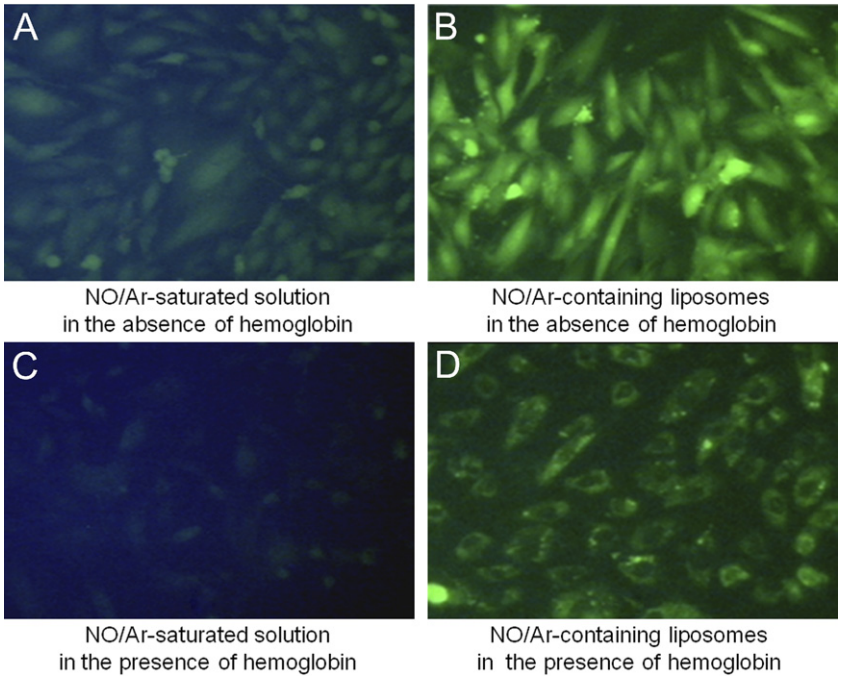

Figure 4 **Cellular Uptake of NO**

Representative DAF-2DA fluorescence images of vascular smooth muscle cells treated with **(A)** NO/Ar-saturated mannitol solution in the absence of hemoglobin, **(B)** NO/Ar (1:9)-containing liposomes in the absence of hemoglobin, **(C)** NO/Ar (1:9)-saturated mannitol solution in the presence of hemoglobin, and **(D)** NO/Ar (1:9)-containing liposomes in the presence of hemoglobin. DAF-2DA = diaminofluorescein-2 diacetate; other abbreviations as in Figure 1.

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delivered to cells was 7-fold greater with NO/Ar-ELIP than with NO/Ar-saturated mannitol solution (Fig. 3).

Because hemoglobin is the primary scavenger of NO in the circulation, which drastically reduces the bioavailability of NO to target tissues, we evaluated the delivery of NO/Ar-ELIP in the presence of hemoglobin (Figs. 4C and 4D). Cellular uptake of NO was visualized by DAF-2DA fluorometric imaging. In the absence of hemoglobin, both the NO/Ar-saturated mannitol solution and the NO/Ar-ELIP efficiently delivered NO to VSMCs (Figs. 4A and 4B). The fluorescence signal was stronger inside cells incubated with NO/Ar-ELIP than with the NO/Ar-saturated mannitol solution. In the presence of hemoglobin, NO delivery to cells from NO/Ar-saturated mannitol solution was poor (Fig. 4C), whereas, that from NO/Ar-ELIP was effective, as indicated by the bright cellular fluorescence (Fig. 4D).

Effects of local delivery of NO/Ar-ELIP on the development of neointimal hyperplasia in balloon-injured arteries. The combination of balloon denudation of the carotid arteries and cholesterol feeding induced extensive neointimal hyperplasia and luminal narrowing relative to the uninjured arteries (Figs. 5A and 5B). The Ar-ELIP did not attenuate the hyperplastic reaction in balloon-injured arteries (Fig. 5C). However, NO/Ar-ELIP was able to inhibit the hyperplastic response in balloon-injured arteries (Figs. 5D and 6). Intima/media thickness ratios decreased from 1.1 ± 0.39 in injured control animals without any specific treatment to 0.5 ± 0.09 in animals treated with NO/Ar-ELIP (Fig. 6A). Similarly, the intimal area was reduced from 0.4 ± 0.09 mm^2 in the injured control animals to 0.2 ± 0.07 mm^2 in the NO/Ar-ELIP-treated animals ($n = 9$, $p < 0.05$) (Figs. 6B and 5B vs. 5D). Ar-ELIP had no effect on the intima/media thickness ratio or intimal area. The decrease in intimal area induced by NO/Ar-ELIP accounted for the decrease in the arterial wall thickness (Table 1).

Discussion

Impaired endothelial NO production is implicated in many cardiovascular diseases, such as essential hypertension, stroke, coronary artery disease, atherosclerosis, platelet aggregation and restenosis after percutaneous transluminal coronary angioplasty, and ischemia/reperfusion injury (15,16). The administration of exogenous NO can potentially supplement NO to the arterial wall and has been explored during the past decade as a therapeutic agent (16). This study has demonstrated that encapsulation of a bioactive gas, NO, into ELIP can be achieved with a freezing under pressure technique. The resultant NO-ELIP has a biphasic release profile. The amount of releasable NO from ELIP can be modulated by mixing it with Ar, thus prolonging NO delivery for in vivo applications. We clearly demonstrate from in vitro, ex vivo, and animal models that liposomal encapsulation of NO protects the payload against NO scavenging by hemoglobin and that effective NO delivery by NO-ELIP inhibits intimal hyperplasia in injured arterial segments. This is the first study to describe liposo-

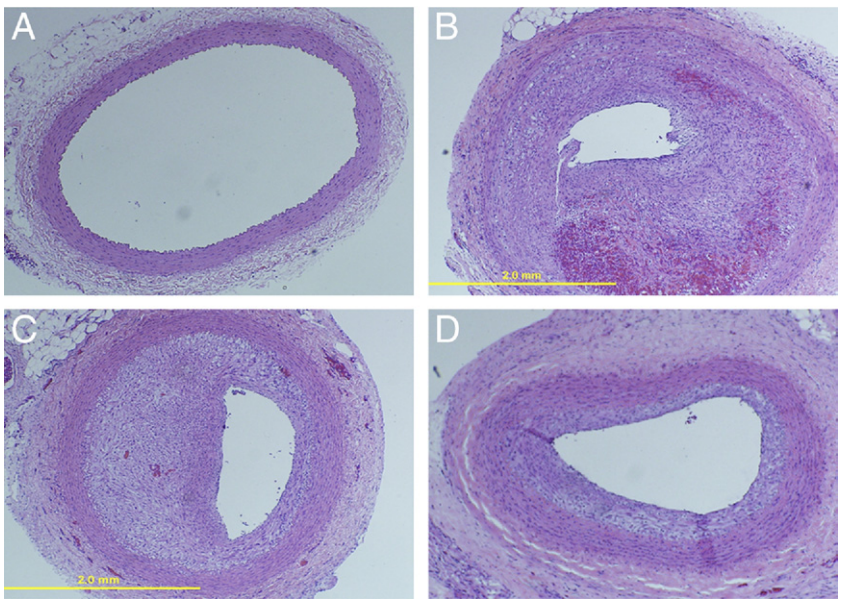

Figure 5 NO/Ar-ELIP Effect on Inhibition of Neointimal Hyperplasia

Representative histological sections of **(A)** normal common carotid artery (no balloon injury), **(B)** common carotid artery 14 days after balloon injury, **(C)** common carotid artery 14 days after balloon injury treated with Ar-ELIP, and **(D)** common carotid artery 14 days after balloon injury treated with NO/Ar (1:9)-containing liposomes (hematoxylin and eosin ×400). Abbreviations as in Figure 1.

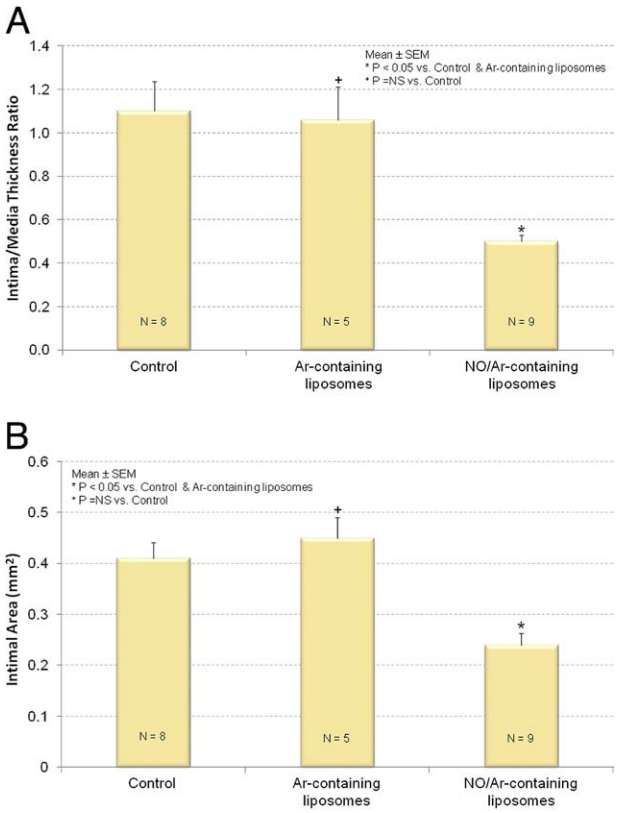

Figure 6 **Quantitation of Neointimal Hyperplasia**

(A) Intima/media thickness ratio and **(B)** intimal area of injured common carotid arteries: control **(left)**; treated with Ar-ELIP **(middle)**; and treated with NO/Ar (1:9)-containing liposomes **(right)**. Abbreviations as in Figure 1.

mal encapsulation of a bioactive gas for controlled release with in vivo biologic effects. We used NO as a model gas in the current study, and this new methodology of gas encapsulation into liposomes can be applied to other therapeutic gases.

Liposome for bioactive gas entrapment and delivery. Pathological changes to vascular wall have been observed in cardiovascular diseases such as atherosclerosis (17). Numerous pharmaceutics have been investigated to treat or prevent atherosclerosis. Gases can be thought of as small molecules that readily cross the endothelial barrier (18). The obstacles of using bioactive gases for the treatment of other vascular conditions have been related to the lack of suitable administration methods and potential adverse effects as the result of systemic delivery. With our new methodology, we have demonstrated that local delivery of NO-ELIP into carotid arteries can inhibit intimal hyperplasia in a balloon-injured arterial model.

The technique of freezing under pressure is effective in encapsulating precise amounts of NO in liposomes. With this technique, we postulate that the increased concentrations of NO dissolved in solution provide a means for the entrapment of NO in the hydrophobic bilayer of NO-ELIP during the freezing and thawing process (13). Nitric oxide is a colorless and odorless gas that readily reacts with oxygen to form NO_2 gas, which is toxic to cells. In our formulations, we took special precautions to prevent NO from reacting with oxygen because this interaction would have resulted in the formation of toxic NO_2. This precaution included mixing NO with an inert gas, Ar, and deoxygenating all gases by passing them through a saturated solution of sodium hydroxide.

The release of NO from NO-ELIP is rapid, and one-half of the payload is released within the first 10 min followed by an extended period of slower release over at least 8 h (Fig. 1). Such a release profile is highly desirable when treating acute arterial injury. In contrast, the other ELIP formulation that contains a mixture of NO and Ar has slower NO release kinetics that may be suitable for clinical settings when chronic low level delivery is desirable.

Sixty percent of the lipid shell of NO-ELIP is comprised of positively charged phospholipids, which are known to facilitate attachment of liposomes and other lipid particles to negatively charged endothelial surfaces and exposed extracellular matrix in damaged arteries. We have previously demonstrated that our ELIP formulations are suitable for carrying air and/or a variety of drugs such as papaverine hydrochloride (19), azithromycin (20), tissue plasminogen activator (21,22), and rosiglitazone (23). In the current study, we demonstrated that 6 μl of NO can be encapsulated into 1 mg of lipids in NO-ELIP by using a relatively low pressure. We have also previously demonstrated that surface modifications of ELIP with antibodies can target ELIP to inflamed arterial walls and localize the therapeutics (10). The NO-ELIP are echogenic and provide a means for monitoring in the circulation and delivery of NO-ELIP with contrast-enhanced ultrasound imaging. As needed, a rapid but controlled payload release from ELIP can be achieved by the use of Doppler ultrasound at a specific site (24). All the listed techniques have the potential to maxi-

Table 1 **Morphometric Analysis of the Carotid Arteries 14 Days After Balloon Injury**

Treatment Group	Intimal Area (mm^2)	Intimal Depth (mm)	Medial Area (mm^2)	Medial Depth (mm)	I/M Ratio	I/(I+M)
Injury alone (n = 8)	0.41 ± 0.03	0.18 ± 0.02	0.42 ± 0.03	0.12 ± 0.01	1.1 ± 0.14	0.5 ± 0.04
Ar-liposomes (n = 5)	0.45 ± 0.04	0.15 ± 0.01	0.44 ± 0.04	0.13 ± 0.004	1.1 ± 0.15	0.5 ± 0.08
NO/Ar-liposomes (n = 9)	0.24 ± 0.02†	0.09 ± 0.01*	0.45 ± 0.03	0.12 ± 0.01	0.5 ± 0.03†	0.3 ± 0.03†

Values are mean ± SEM. *$p < 0.05$ versus injury alone and Ar-containing liposomes. †$p < 0.005$ versus injury alone and Ar-containing liposomes.
AR = argon; I/M = intima/media; NO = nitric oxide.

mize the effects of drugs and/or bioactive gases in a localized manner.

We have demonstrated that local administration of NO-ELIP is effective in reducing neointimal hyperplasia in injured carotid arteries in hyperlipidemic rabbits. Such a model resembles the clinical situation of balloon angioplasty in coronary and other vascular beds. The in vivo effect of inhaled NO on neointimal hyperplasia has been inconsistent. Lee et al. (25) have described a beneficial effect of inhaled NO in reducing neointimal formation in balloon-injured carotid arteries in rats. Others (26), however, have been unable to demonstrate NO effects in the pulmonary vasculature.

Hemoglobin binding to inhaled NO is likely responsible for the inability of NO to reach the intended vascular beds. The affinity of NO for hemoglobin is 1,500 times greater than that of carbon monoxide. It has been demonstrated that red blood cells, because of their high hemoglobin content, can reversibly bind, transport, and release NO within the cardiovascular system (27). In Figure 4, the NO/Ar-saturated mannitol solution was less efficient in delivering NO to VSMCs than the NO/Ar-ELIP; both methodologies were rendered less effective for NO delivery to VSMC in the presence of hemoglobin. However, Figures 4A and 4D appear similar, and there is consistency between the fluorescent enhancement of Figures 4B and 4D. This finding clearly demonstrates the ability of the liposomes to protect NO from hemoglobin scavenging. This mixture of NO/Ar further modulates the rate of NO delivery into cells. This experimental condition consisting of the presence of hemoglobin mimics local NO delivery by NO/Ar-ELIP in vivo, and animal experiments also substantiate the biologic effects of NO/Ar-ELIP when delivered locally in balloon-injured arteries (28).

Study limitations. To demonstrate NO release, we delivered NO-ELIP (without targeting) to an occluded artery locally. This method of delivery may mimic certain interventions such as angioplasty of an occluded coronary or carotid artery but may not simulate physiologic flow conditions when arterial flow is present. Although we have demonstrated the protective effects of NO encapsulation into liposomes for preventing NO scavenging by hemoglobin, we assume that continuous blood flow will probably increase the scavenging effect of NO by hemoglobin. This may be overcome by local delivery of NO-ELIP, targeting of the NO-ELIP, and ultrasound-enhanced NO release. Local delivery of antiproliferative or immunosuppressive agents immediately after angioplasty has been successful in clinical trials and has been proven more efficient compared with systemic drug delivery (29).

Recently, Cyrus et al. (30) have developed a "contact facilitated drug delivery" methodology to incorporate $\alpha_v\beta_3$-targeted nanoparticles containing rapamycin for targeting and content release through fusion of the lipid membrane with the targeted cell membrane. This methodology could also be useful in targeting and release of our NO-ELIP. In another study, we have also found that local ultrasound application enhances NO delivery to the arterial wall under physiological flow conditions, demonstrating another potential methodology to increase NO delivery.

The spontaneous release pattern, in which release is initially rapid and then later much slower, may limit the amount of gas deliverable to targeted sites, with the consequence of reducing localized delivery and increasing systemic exposure. Further modifications of the lipid shell composition as to make it less permeable and reduce spontaneous gas release may overcome this problem. Surface modifications of NO-ELIP such as the addition of polyethylene glycol and antibodies to the lipid shell to prolong circulation duration and provide specific targeting mechanisms were not evaluated in this study.

Despite a lack of sophistication of our initial approaches, marked in vivo effects were realized which induced inhibition of intima/media ratio by 40% by NO-ELIP in injured arteries compared with Ar-ELIP or no treatment. Therapeutic effects should be improved by coencapsulating NO with other NO donors or synthetic genes involved in NO synthesis, although the benefit of such procedures must be assessed by future research.

Conclusions

A new technique has been described for encapsulating NO into liposomes for bioactive gas delivery. Such formulations have the desirable properties of high payload concentration, modifiable gas release profiles, sufficient protection of the NO payload against exogenous scavengers, and excellent payload delivery to cells for biologic effects. With future formulation modifications, this methodology may expand the repertoire of NO donors in the payload, providing greater stability, as well as site-specific triggered delivery with ultrasound, to maximize bioactive effect at the local tissue level.

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