Detection of Retained Microbubbles in Carotid Arteries With Real-Time Low Mechanical Index Imaging in the Setting of Endothelial Dysfunction

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OBJECTIVES We sought to determine if intravenously injected microbubbles would be retained by the carotid arteries (CAs) in the setting of endothelial dysfunction (ED) using a linear transducer equipped with a low mechanical index pulse sequence scheme (PSS).

BACKGROUND Microbubbles normally pass freely through large and small vessels but are retained in regions with ED. New high-frequency low mechanical index PSS can potentially be utilized to image these retained microbubbles.

METHODS Intravenous albumin- and lipid-encapsulated microbubbles were administered in seven pigs while imaging the CAs before and after a 20% intralipid infusion to induce hypertriglyceridemia. The degree of microbubble retention was quantified by measuring endothelial acoustic intensity (AI) after clearance of free-flowing microbubbles. Microbubble adherence was also evaluated after selective balloon injury of the CAs. The CA diameter responses to acetylcholine were quantified.

RESULTS After induction of hypertriglyceridemia, adherence of albumin-encapsulated microbubbles was visually evident in all CAs, and endothelial AI increased significantly (p < 0.001 compared with baseline). The CA responses to acetylcholine went from vasodilation at baseline to vasoconstriction during hypertriglyceridemia. Endothelial AI also increased in the balloon-stretched vessels (p < 0.01 compared with uninjured vessels) after albumin-encapsulated microbubble injection, with a ring of microbubbles selectively adhering to the injured segment. This retention was not observed with lipid-encapsulated microbubbles. Scanning electron microscopy confirmed that albumin-coated microbubbles adhered to endothelial cells.

CONCLUSIONS Retention of intravenously injected albumin microbubbles occurs in the setting of both global and regional ED in large vessels and can be noninvasively imaged with high-frequency low mechanical index PSS. (J Am Coll Cardiol 2004;44:1036–46) © 2004 by the American College of Cardiology Foundation

Endothelial dysfunction (ED) is considered one of the earliest events in the process of atherosclerosis (1) and is associated with most cardiovascular risk factors, including diabetes mellitus, hyperlipidemia, smoking, and hypertension (2–4). Endothelial dysfunction also appears to be the first identifiable functional abnormality observed with a high-fat diet, balloon- or stent-induced arterial injury, reperfusion injury, or transplant rejection (5–8).

Because ED is an early reversible risk factor in the development of atherosclerosis, its identification is an important clinical end point. Current methods for detecting ED are the evaluation of endothelium-dependent, flow-mediated dilation, or response to an endothelium-dependent vasodilator infusion, usually assessed by ultrasound or plethysmography of the brachial artery (5,6). It is known that in patients with coronary artery disease there is correlation between impaired endothelium-dependent vasodilation in the brachial artery and the presence of ED in the coronary arteries (7). However, a noninvasive direct method to visualize ED has not been developed.

Intravenously injected perfluorocarbon-filled microbubbles are currently indicated for the improvement of endocardial border resolution. These microbubbles exhibit rheologic properties similar to those of red blood cells and normally pass freely through the large and small vessels (8). However, several investigators have demonstrated that microbubbles are retained in the microvasculature when ED exists (9–12). It is unknown whether regional or global arterial ED would also result in retention of these microbubbles.

One of the major limitations for imaging a small number of microbubbles retained along the endothelial border is that they are easily destroyed by the interrogating beam. However, a new low mechanical index pulse sequence scheme (PSS) has been developed which is sensitive enough to detect nonlinear behavior from small numbers of microbubbles with minimal destruction while simultaneously canceling background linear signals from tissue (13). In this study, we utilized a high-frequency version of this PSS to determine whether endothelial retention of microbubbles occurs with regional or global arterial ED.
METHODS

Animal preparation. The study was approved by the Institutional Animal Care and Use Committee of the University of Nebraska Medical Center and conformed to the Position of the American Heart Association on Research Animal Use. Nine pigs weighing 36 ± 2 kg were sedated, endotracheally intubated, and placed under general anesthesia using isoflurane. In each animal, 7-F catheters were placed in both femoral veins for administration of microbubbles and intralipid emulsion and for withdrawal of blood samples. Both femoral arteries were also cannulated for hemodynamic monitoring and for placing an 8-F guide catheter into the proximal left carotid artery (CA). All pigs were treated with 40 mg of Ketorolac intravenously to prevent pulmonary hypertensive responses to microbubbles, which occur exclusively in this species (14), and ventilated with supplemental oxygen during the microbubble injections.

Evaluation of endothelial function. Endothelial function was evaluated by measuring the responses of the diameter of the CAs to intra-arterial infusion of the endothelium-dependent vasodilator acetylcholine. Local infusions of acetylcholine normally result in either no change in vessel diameter or mild vasodilation, whereas abnormal responses produce endothelin-mediated vasoconstriction (15). Longitudinal sectional images of the vessel were obtained with a standard 7–4 MHz linear transducer (HDI 5000, Philips Medical Systems, Bothell, Washington) at a mechanical index of 0.8. After adequate transcutaneous imaging of the CA with this transducer, the skin overlying the vessel was marked to permit monitoring of the vessel at one constant location. Also, a radiopaque marker was positioned at this site to permit its adequate identification by angiography. The CAs were scanned longitudinally, and their diameters were measured from two-dimensional images, from the anterior to the posterior inner borders of the vessels at end-diastole, coincident with the R wave on a continuously recorded electrocardiogram. Mean flow velocity was obtained with pulsed Doppler by placing the sample volume in the center of the artery. The average of three cardiac cycles was used for analysis.

After baseline CA measurements, acetylcholine was infused to achieve estimated intra-arterial concentrations of $10^{-7}$ and $10^{-6}$ mol/l, at 10-min intervals through the 8-F catheter. The flow in the CA obtained by ultrasound was used to determine the molar concentrations of acetylcholine to be infused, as follows:

$$Q (\text{ml/min}) \times M_{\text{Ach}} \times 182 \text{ g Ach/mol/l} \times 1.0 \min /\text{ml}$$

where 1.0 ml/min was the infusion rate for acetylcholine; $Q = \text{CA flow calculated by the product of CA area and mean velocity};$ and $M_{\text{Ach}} = \text{molar concentration of acetylcholine desired (mol/l)} (16).$

The response of CA diameter to acetylcholine was considered as the highest variation from baseline during the $10^{-7}$ or $10^{-6}$ mol/l infusion.

Carotid imaging of retained microbubbles. The evaluation of both albumin- and lipid-encapsulated microbubble transit in the CAs was performed with a novel 15L8 linear array transducer equipped with a low mechanical index PSS (Contrast Pulse Sequencing; Siemens Acuson Sequoia, Mountain View, California) operating at 7 MHz. This technique transmits pulses of alternating polarity and amplitude to detect nonlinear responses from microbubbles while simultaneously canceling the linear responses from tissue (13).

Perfluorocarbon-exposed sonicated dextrose albumin (PESDA) microbubbles were injected as a bolus of 0.5 ml followed by a 10-ml saline flush into the femoral vein. The PESDA was prepared according to the method previously described (17). Briefly, a mixture of three parts 5% dextrose and one part 5% human serum albumin (total, 16 ml) was added to 8 ml defluorobutane gas, hand-agitated, and then sonicated with an electromechanical sonicator (Heat Systems Inc., Farmingdale, New York) for 70 ± 5 s. The resulting microbubble size by hemocytometry was 4.6 ± 0.4 μm, and mean concentration measured by a Coulter counter was $1.4 \times 10^9$ bubbles/ml.

Lipid-encapsulated microbubbles (Definity; Bristol-Myers Squibb Medical Imaging Inc., North Billerica, Massachusetts) were injected as a bolus of 0.05 ml followed by a 10-ml saline flush into the femoral vein. Definity microbubbles are composed of octafluoropropane gas encapsulated in a lipid shell with a polyethylene glycol spacer on its surface that inhibits any complement bound to the microbubbles from binding to the vascular endothelium (18,19). The reported diameter of Definity is 1.1 to 3.3 μm, and the mean concentration is $1.2 \times 10^{10}$ bubbles/ml. Therefore, we reduced the dose of Definity microbubbles 10-fold to match the concentration of PESDA microbubbles injected.

Carotid arteries were imaged in both the transverse and longitudinal sectional views, in the same position used for the evaluation of diameter responses to acetylcholine. The depth, power output, gain, and compression settings were initially adjusted to obtain adequate detection of the microbubbles by real-time imaging and kept constant throughout the examination. The mechanical index used was 0.3, and the frame rate was 24 Hz. Retained microbubbles were
defined qualitatively as microbubbles adherent to the arterial wall which persisted in one location at the vessel luminal border late after injection. Examinations were recorded on videotape, and the images were digitally stored for subsequent analysis.

Quantification of the contrast acoustic intensity (AI) was performed by analyzing digitally acquired cine loops with quantitative contrast software (CUSQ 1.3, Siemens Acuson, Mountain View, California). Transverse sectional views of the CAs were used for this analysis. To determine the AI of microbubbles retained to the arterial endothelium, we performed a simultaneous quantification of the entire lumen AI and the central lumen AI. The endothelial AI was defined as the difference between the entire lumen AI minus the central lumen AI (Fig. 1A).

Quantification of the entire lumen AI (encompassing the endothelial border and central lumen) was obtained by placing a region of interest (ROI) just inside the lumen border on the transverse carotid image, thus avoiding the analysis of contrast signals coming from the vasa vasorum. A second ROI, with a diameter 20% smaller than the larger ROI, was then placed in the central lumen of the vessel (Fig. 1A). The mean AI of both ROI was measured simultaneously. For each sequence of images at baseline, post intralipid infusion and post balloon injury, 50 consecutive frames in the late period following microbubble injections after clearance of free-flowing MB were analyzed (Fig. 1B). A typical bolus injection of microbubbles resulted in a 20- to 25-s period of complete vessel opacification, followed by a washout period. Therefore, the sequence of

Figure 1. (A) Example of the region of interest (ROI) used for the measurements of contrast acoustic intensity (AI) of the entire lumen (white line) and central lumen (yellow line). (B) Two-dimensional (2D) imaging of a normal carotid artery in the transverse view and with pulse sequence scheme (PSS) before intravenous microbubbles (MB) injection showing no background imaging, early after MB injection showing the complete luminal vessel opacification, and late after MB injection showing few MB in the central lumen. The time-intensity curve demonstrates the typical quantification of the mean AI in both the entire lumen ROI and the central lumen ROI. The late 50 frames after clearance of free-flowing MB were chosen for the calculation of endothelial AI, defined as the difference between the mean entire lumen AI minus the central lumen AI (see text for details).
frames chosen for quantification of endothelial AI was initiated 30 s after the first detection of microbubbles in the CA. Endothelial AI was expressed as the mean difference between the entire lumen AI and the central lumen AI in the 50 analyzed frames. The results of AI were expressed in units (range 0 to 100).

**Study protocol. PHASE I: INDUCTION OF HYPERTRIGLYCERIDEMIA.** This portion of the study involved seven pigs. After baseline microbubble injections and measurements of endothelial function, transient hypertriglyceridemia was induced by a continuous intravenous infusion of 20% intralipid (Fresenius Kabi Clayton, L.P., Clayton, North Carolina), at a rate of 0.3 ml/kg/min, for 20 min (5,20). Measurements of serum levels of triglycerides and cholesterol were performed before and after intralipid infusion. In a similar way, the serum levels of complement (C3) were measured at baseline, before and after microbubble injection, and again during hypertriglyceridemia. The CA responses to intra-arterial acetylcholine infusion, and imaging of CAs after albumin- and lipid-encapsulated microbubble injections, were repeated 30 min after intralipid infusion. Three pigs were euthanized at this phase of the study for scanning electron microscopy (SEM) verification of microbubble binding.

Serum was also obtained before and after induction of hypertriglyceridemia for flow cytometry, to determine whether hypertriglyceridemia influenced serum complement attachment to the microbubble surface. For this procedure, 0.7 × 10⁶ PESDA microbubbles were combined with 0.5 ml of pig serum, incubated at 37°C for 5 min, and washed twice. The serum-exposed microbubbles were then combined with anti-human C3b murine monoclonal antibody (Gamma Biologicals Inc., Houston, Texas) for 20 min, washed twice, and subsequently combined with a R-phycoerythrin conjugated secondary antibody (Becton Dickinson Pharmigen, San Jose, California) for 20 min. Aliquots of 0.7 × 10⁶ PESDA microbubbles were also incubated with immunoglobulin G control antibody (Alpha Diagnostic, San Antonio, Texas) and R-phycoerythrin conjugated secondary antibody, or only with R-phycoerythrin conjugated secondary antibody. Microbubbles were then analyzed on a FACSCalibur (Becton Dickinson, San Jose, California) to generate graphs of red fluorescent intensity.

**PHASE II: BALLOON-STRETCHING OF THE CAs.** This portion of the study involved six pigs. After a single intravenous bolus of heparin (100 USP U/kg), baseline carotid angiography was performed using injections of iohexol 300 (Omnipaque; Nycomed, Princeton, New Jersey), and the vessel diameter was determined with a manual caliper. A 40- to 60-mm segment of the left CA was stretched using an oversized balloon dilation catheter (Guidant, Advanced Cardiovascular Systems, Temecula, California) that measured 140% of baseline angiographic vessel diameter, as previously described (14). In one pig there was dissection and occlusion of the left CA during the procedure. Dilation of the right CA was subsequently performed and used for analysis. In the remaining five pigs, the uninjured right CA served as control.

Intravenous PESDA and Definity injections were repeated for imaging of microbubble transit in both the balloon-injured and the control CAs within 1 h of arterial injury. The CA responses to local acetylcholine infusions were also re-evaluated. At the end of the protocol, the pigs were euthanized, and sections of both CAs were analyzed by SEM. In five of these pigs, a final 0.5-ml, albumin-encapsulated microbubble injection was performed without any ultrasound imaging 15 min before the pigs were euthanized.

**PATHOLOGIC ANALYSIS OF THE CA.** The balloon-injured, hypertriglyceridemic, and control CAs were excised, washed in 0.9% saline solution, and fixed in 10% formalin for a minimum of 24 h. Histologic analysis was performed in sections of these vessels to assess the extent of injury. For SEM, the CAs were longitudinally sectioned and dehydrated through a series of ethanol concentrations increasing from 50% to 100%. The specimens were then immersed in Freon 113, critical point dried, mounted on aluminum stubs, and sputter-coated with gold (Polaron E5100, Polaron Inc., Watford, United Kingdom).

To determine the appearance of microbubbles by SEM, we also evaluated an in vitro preparation of PESDA. The microbubbles were fixed in 10% formalin, placed over a 0.22-μm Millipore filter (Millipore Corporation, Bedford, Massachusetts), and submitted to the same processing as described for the CA specimens. Scanning electron microscopy was performed using a Philips 515 system (Philips Inc., Eindhoven, the Netherlands).

**Statistical analysis.** Continuous data are expressed as mean ± SD. Comparisons of endothelial AI before and after procedures were performed by a paired t test. The CA diameter responses to acetylcholine infusion were compared by a Wilcoxon signed rank test. An unpaired t test was used for the comparison of endothelial AI in the balloon-injured and control vessels. Triglyceride and cholesterol levels at baseline, immediately after intralipid infusion, and 2 h after intralipid infusion were compared by repeated measures analysis of variance. A p value <0.05 was considered statistically significant.

**RESULTS**

Complement binding to microbubbles. Flow cytometry revealed complement binding to PESDA microbubbles in the presence of pig serum, which did not occur with the control antibody or with the anti-mouse secondary antibody alone (Fig. 2, curves A and B). The binding of anti-human C3b to PESDA microbubbles was increased during hypertriglyceridemia, expressed as a shift of the mean fluorescent intensity curve to the right (Fig. 2, curves C and D).

**Serum triglyceride and complement levels.** Serum triglyceride and cholesterol levels were low in all pigs at baseline. Intralipid infusion resulted in a significant increase
in the serum triglyceride levels, returning to near baseline values at 2 h after infusion. No significant change was observed in the cholesterol measurements.

Complement levels were normal in all pigs at baseline and remained unchanged after microbubble injection. However, during hypertriglyceridemia there was a significant increase in the serum levels of complement C3 (Table 1).

**CA response to acetylcholine.** The CA response to the acetylcholine at baseline was vasodilation, reflecting normal endothelial function, in all but one pig in which a vasoconstrictive response was obtained. The presence of ED in the remaining pigs after intralipid infusion was confirmed by the CA response to acetylcholine, which went from vasodilation at baseline to vasoconstriction during hypertriglyceridemia \( (p = 0.024) \) (Fig. 3A). In a similar way, a vasoconstrictive response to intra-arterial infusion of acetylcholine was also observed in the balloon-injured CAs (Fig. 3B).

There were no significant differences in the arterial blood pressures or heart rates between baseline, post-intralipid, and post-dilation conditions.

**Carotid imaging of retained microbubbles.** A clear visualization of the PESDA and Definity microbubble transit in real time was obtained by imaging the CAs with the 7-MHz contrast pulse sequencing PSS. No visually evident retention of microbubbles to the arterial endothelium was

<table>
<thead>
<tr>
<th>Table 1. Lipid and Complement Levels at Baseline and Post-Intralipid Infusion</th>
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<tr>
<td><strong>Baseline</strong></td>
</tr>
<tr>
<td>Triglyceride (mg/dl)</td>
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<tr>
<td>Total cholesterol (mg/dl)</td>
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<tr>
<td>Complement C3 (mg/dl)</td>
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*\( p < 0.05 \) compared with baseline and 2 h later. †\( p = \) NS compared with before MB injection. ‡\( p < 0.05 \) compared with baseline. Values are mean ± SD.

MB = microbubble.
detected late (>30 s) after PESDA injections at baseline, except in the pig that exhibited a vasoconstrictive response to acetylcholine. In this pig, retention of PESDA microbubbles to the arterial endothelium could be observed (pig 4) (Fig. 4).

After induction of hypertriglyceridemia, imaging of both right and left CAs before microbubble injections was performed and confirmed no background signals emanating from the endothelium. After intravenous PESDA injection, microbubble retention to the arterial endothelium was evident in both CAs of all pigs. This microbubble retention appeared as numerous bright, circular densities creating a ring of enhancement around the vessel (Fig. 4).

Visual adherence of PESDA microbubbles to arterial endothelium was also observed in all balloon-injured CAs and in none of the control vessels (Fig. 5, Video 1). This retention of microbubbles to the arterial endothelium, however, was not observed with Definity microbubbles, either during hypertriglyceridemia or after balloon injury of the CAs.

Endothelial AI quantification. After intralipid infusion, a significant increase in the endothelial AI was measured in all CAs after PESDA injection, except in the right CA of the pig in which microbubble adherence to the CA wall was detected even at baseline. Baseline endothelial acoustic intensities were less than zero owing to residual microbubbles in the central portion of the lumen. The mean endothelial AI measured late after PESDA injections increased by 1.1 ± 1.2 U during hypertriglyceridemia (p < 0.001) (Table 2). However, there was no difference in the endothelial AI after intravenous Definity when comparing baseline (−1.09 ± 1.03 U) and hypertriglyceridemia (−1.05 ± 1.05 U; p = 0.56). Figure 6 is an illustrative example of time-intensity curves obtained after intravenous PESDA and Definity microbubble injections during hypertriglyceridemia. The analysis of the curves in the late period after
PESDA injection demonstrates that the entire lumen AI was higher than central lumen AI, resulting in a positive value of mean endothelial AI. After Definity injection, the central lumen AI was slightly higher than the entire lumen AI at the same time interval, with a resulting negative value of endothelial AI.

The endothelial AI after PESDA microbubble injections in the balloon-injured vessels was significantly higher than the endothelial AI in the control CAs. No difference in the endothelial AI between these two CAs (balloon-injured vs. contralateral control) was observed with intravenous Definity (Table 2).

SEM. Histologic analysis demonstrated preserved endothelium in the balloon-injured CAs, with minimal endothelium denudation, and no other damage in the media or adventitia layers.

The results of in vitro studies confirmed that microbubbles fixed with 10% formalin were stable and survived the SEM processing. When examined under SEM, microbubbles were characterized as a less electron-dense structure, with sizes ranging from 1 to 5 μm, spherically shaped (differentiating them from the biconcave-shaped erythrocytes), and not exhibiting any of the surface characteristics of hematopoietic cells, such as microvilli or microridges (Fig. 7). These criteria were then used to differentiate the microbubbles from red blood cells or endothelial protusions in the CA specimens.

Scanning electron microscopy of CAs demonstrated evidence of endothelial stretching with minimal denudation in all balloon-dilated vessels, and normal-appearing endothelium in the control vessels. Denudation of the endothelial layer was observed in <5% of the scanned regions of the injured CAs. However, microbubbles were attached to both the denuded areas and the endothelial cells within the stretched segments (Fig. 8A) and were not found in the normal control vessels (Fig. 8B). Scanning electron microscopy also confirmed the presence of microbubbles attached to the otherwise normal-appearing arterial endothelium of all three pigs euthanized during hypertriglyceridemia (Fig. 9).

DISCUSSION

In this study, we were able to detect and quantify microbubble adherence to dysfunctional endothelium using a high-resolution low mechanical index PSS. Using this low mechanical index prevented microbubble destruction and thus minimized any possible damage to the endothelium caused by the ultrasound. Dysfunctional endothelium of the CAs was induced globally by transient hypertriglyceridemia, and then regionally with balloon injury. In both settings, ED in the CAs was confirmed by a vasoconstrictive response to intra-arterial acetylcholine. The retained microbubbles were seen only in CAs that exhibited abnormal endothelial vasomotion.

The term “endothelial dysfunction” refers to a broad alteration in the endothelial phenotype that may lead to the development of atherosclerosis (21). Both traditional and newly recognized cardiovascular risk factors initiate a chronic inflammatory process that is accompanied by a loss of endothelium-dependent vasodilatory and antithrombotic properties. Current diagnostic tests designed to detect ED have centered around the detection of abnormalities in nitric oxide production. However, more recent observations have emphasized the anti-inflammatory properties of the endothelium, and that inflammatory markers such as C-reactive protein may be a mediator in the vasoconstrictive response to agents such as acetylcholine (22). Microbubble binding to endothelium has been con-
sistantly associated with increased endothelial expression of inflammatory markers (9–11). This inflammation process appears to play a role in the downregulation of nitric oxide synthase and augmentation of endothelium-derived vasoconstrictor production (22). Thus, microbubble adherence could indicate the presence of inflammatory markers that

### Table 2. Values of Endothelial Acoustic Intensity, Expressed in Units, at Baseline, During Hypertriglyceridemia, and After Balloon-Stretching of the Carotid Arteries Following Albumin- and Lipid-Encapsulated Microbubble Injections

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Post-Intralipid</th>
<th>Stretched CAs</th>
<th>Control CAs</th>
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<tbody>
<tr>
<td><strong>PESDA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Mean</td>
<td>$-0.90 \pm 0.84$</td>
<td>$0.23 \pm 1.03^*$</td>
<td>$0.68 \pm 1.28$</td>
<td>$-0.53 \pm 0.98^\dagger$</td>
</tr>
<tr>
<td>Range</td>
<td>$-1.29$ to $-0.68$</td>
<td>$-0.50$ to $0.80$</td>
<td>$+0.09$ to $2.31$</td>
<td>$-1.11$ to $0.06$</td>
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<tr>
<td><strong>Definity</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>$-1.09 \pm 1.03$</td>
<td>$-1.05 \pm 1.05$</td>
<td>$-1.16 \pm 0.99$</td>
<td>$-1.20 \pm 1.04$</td>
</tr>
<tr>
<td>Range</td>
<td>$-2.21$ to $-0.23$</td>
<td>$-1.54$ to $-0.48$</td>
<td>$-1.60$ to $-0.44$</td>
<td>$-1.63$ to $-0.59$</td>
</tr>
</tbody>
</table>

$^\dagger p < 0.001$ compared with baseline; $^\dagger p < 0.01$ compared with stretched CA. Values are mean ± SD.

CAs = carotid arteries; PESDA = perfluorocarbon-exposed sonicated dextrose albumin.

**Figure 6.** Example of time-intensity curve showing the difference between the mean acoustic intensity (AI) of the entire lumen (dark line) and the central lumen (gray line) regions (A), and transverse images of carotid artery after PESDA and Definity microbubbles (MB) injection in the setting of hypertriglyceridemia (B). Note the retention of PESDA MB around the vessel, not seen with Definity. Amplification of the curves in the late period after PESDA MB injection (arrow) demonstrates a resulting positive value of mean endothelial AI. On the other hand, after Definity MB injection the central lumen AI was slightly higher than the entire lumen AI with a resulting negative value of endothelial AI.
initiate the atherosclerotic process and mediate the loss of appropriate vasodilator responses.

This study is the first to demonstrate direct visualization of microbubble binding to dysfunctional endothelium induced by both a local arterial injury and systemic hypertriglyceridemia. Others have demonstrated in animal models that microbubbles specifically targeted to adhesion mole-
cules may be necessary to concentrate retained microbubbles in sufficient quantity that they could be visualized with ultrasound (11,12). Our success with nontargeted micro-
bubbles most likely results from the sensitivity of the contrast pulse sequence imaging scheme in detecting even low concentrations of microbubbles and from the use of a higher frequency transducer, which permitted a higher axial

![Figure 7](image1)

**Figure 7.** The PESDA microbubbles fixed with formalin and examined under scanning electron microscopy (SEM) using a 0.22-μm Millipore filter, at (A) low magnification (bar = 10 μm; magnification 1,420×) and (B) high magnification (bar = 10 μm; magnification 7,400×). Under SEM the microbubbles were characterized as less electron-dense structures of different sizes, spherically shaped and not exhibiting any other surface features of hematopoietic cells.

![Figure 8](image2)

**Figure 8.** High magnification (bar = 10 μm; magnification 1,420×) scanning electron microscopy pictures (left panels) and their respective low mechanical index pulse sequence scheme (PSS) images (right panels). Panel A demonstrates the presence of retained microbubbles in the endothelium detected by PSS in the left injured carotid artery, and Panel B reveals the absence of microbubbles in the endothelium in the control right side. Scanning electron microscopy revealed sites of injury with endothelial denudation and attachment of microbubbles (black arrows) to the denuded endothelium only in the injured vessel (A) and normal-appearing endothelium in the control vessel (B).
resolution image of the endothelial surface in the transverse plane.

**Detection of both global and regional ED using intravenous microbubbles.** Endothelial dysfunction has been observed with transient hypertriglyceridemia induced by an intravenous or oral fat load (23,24). In our study, the presence of hypertriglyceridemia resulted in albumin-encapsulated microbubble retention in anatomically normal appearing CAs. These data indicate that exposure of interstitial matrix beneath the endothelium is not necessary for binding. We also observed direct binding of albumin-encapsulated microbubbles to intact endothelial cells within the balloon-injured segment of the CAs. Others have observed albumin- and lipid-encapsulated microbubble binding to activated neutrophils and monocytes along the inflamed or injured venular endothelial surface (25,26). The absence of such an observation in our study by SEM indicates that a direct interaction between an endogenous receptor on the endothelium cell surface and the microbubble resulted in binding. The only known molecule within serum which binds to untargeted microbubbles is complement (18). An increase in complement receptor density has been observed with acute endothelial injury induced by hypoxia, resulting in increased C3b deposition (27). The C3 levels are augmented with both acute and chronic elevations in serum triglycerides (28,29). With hypertriglyceridemia, the increased complement on the surface of the microbubbles could have increased their binding to complement receptors already on the surface of endothelial cells (30). This would also explain why the lipid-encapsulated microbubbles did not appear to bind in this same setting. Although they also take up complement within serum, they contain a polyethylene glycol spacer that prevents complement-mediated binding to the endothelium (18).

**Study limitations.** The ring of contrast enhancement around the vessel lumen during hypertriglyceridemia and after balloon injury was considered to reflect retained microbubbles to the arterial endothelium. One could argue that this signal could be coming from microbubbles flowing through the vasa vasorum in the arterial wall. However, we did not observe any increase in endothelial AI after lipid-encapsulated microbubbles in any of these situations. Although the Definity microbubbles were smaller, they still produced excellent vessel lumen opacification and would be expected to produce an increase in signal from the vasa vasorum. Secondly, we utilized the outline from the complete opacification of the border between lumen and endothelium early after injection of microbubbles to differentiate the bubbles present in the vessel lumen from those in the immediate peri-luminal area. This also allowed us to put the ROI exactly inside the vessel lumen for quantification of AI.

It has already been demonstrated that the acoustically driven diffusion of gas from the microbubbles is a slower mechanism of bubble destruction, and the rate of this diffusion increases during insonation even when using a low mechanical index. This produces changes in the microbubble diameter on the time scale of the acoustic pulse length (31) and may have caused some microbubble destruction even with the low mechanical index PSS used in this study. This may have prevented the accurate quantification of the number of adherent microbubbles in our study. As the mean size of Definity microbubbles is smaller than PESDA, acoustically driven diffusion from these microbubbles may have resulted in a more rapid loss of signal, and played a role in our observation that retention of microbubbles to the arterial endothelium was not detected after Definity injections.

The low endothelial AI values that we observed reflect the small retention fraction of microbubbles that actually adhere to the dysfunctional endothelium. However, the values of endothelial acoustic intensities under resting conditions were all less than zero, indicating that even late after injection there is still a slightly greater AI from a few residual bubbles in the central lumen. Furthermore, the mean value and range of values for endothelial AI remained less than zero at all stages of the study for intravenous Definity. However, the values for intravenous PESDA in the setting of balloon-induced ED all ranged greater than...
zero (range 0.09 to 2.31 U). Thus, we could both visualize retained microbubbles and quantify a significant change in AI despite the low retention fraction of microbubbles to the endothelium.

Although we observed microbubble retention with acute hypertriglyceridemia in this animal model, we do not know if a similar phenomenon would be observed in humans with chronic or acute hyperlipidemia. Further investigation in this area is necessary.

CONCLUSIONS

A high-frequency linear transducer with a contrast-sensitive PSS was able to image retention of albumin-encapsulated microbubbles in dysfunctional carotid arterial endothelium. Although ED during transient hypertriglyceridemia had already been demonstrated by indirect measurement of vasoreactivity of the brachial artery by ultrasound, this is the first study to visualize retained arterial microbubbles in the setting of ED. Given the critical role of ED and inflammation in the pathogenesis of atherosclerosis and its close relationship with acute coronary syndromes, the detection of endothelial microbubble retention in vivo after intravenous injection of microbubbles could be a powerful clinical tool to identify the presence of vascular inflammation and serially follow it during treatment.

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