

Monosialoganglioside-Containing Nanoliposomes Restore Endothelial Function Impaired by AL Amyloidosis Light Chain Proteins

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Background—Light chain amyloidosis (AL) is associated with high mortality, especially in patients with advanced cardiovascular involvement. It is caused by toxicity of misfolded light chain proteins (LC) in vascular, cardiac, and other tissues. There is no treatment to reverse LC tissue toxicity. We tested the hypothesis that nanoliposomes composed of monosialoganglioside, phosphatidylcholine, and cholesterol (GM1 ganglioside-containing nanoliposomes [NLGM1]) can protect against LC-induced human microvascular dysfunction and assess mechanisms behind the protective effect.

Methods and Results—The dilator responses of ex vivo abdominal adipose arterioles from human participants without AL to acetylcholine and papaverine were measured before and after exposure to LC (20 $\mu\text{g}/\text{mL}$) with or without NLGM1 (1:10 ratio for LC:NLGM1 mass). Human umbilical vein endothelial cells were exposed for 18 to 20 hours to vehicle, LC with or without NLGM1, or NLGM1 and compared for oxidative and nitrative stress response and cellular viability. LC impaired arteriole dilator response to acetylcholine, which was restored by co-treatment with NLGM1. LC decreased endothelial cell nitric oxide production and cell viability while increasing superoxide and peroxynitrite; these adverse effects were reversed by NLGM1. NLGM1 increased endothelial cell protein expression of antioxidant enzymes heme oxygenase 1 and NAD(P)H quinone dehydrogenase 1 and increased nuclear factor, erythroid 2 like 2 (Nrf-2) protein. Nrf-2 gene knockdown reduced antioxidant stress response and reversed the protective effects of NLGM1.

Conclusions—NLGM1 protects against LC-induced human microvascular endothelial dysfunction through increased nitric oxide bioavailability and reduced oxidative and nitrative stress mediated by Nrf-2-dependent antioxidant stress response. These findings point to a potential novel therapeutic approach for light chain amyloidosis. (*J Am Heart Assoc.* 2016;5:e003318 doi: 10.1161/JAHA.116.003318)

Key Words: amyloid • endothelium • nanotechnology • oxidant stress

Light chain amyloidosis (AL) is associated with high mortality and morbidity resulting from clonal overproduction of amyloidogenic light chain proteins (LC) that misfold and deposit in various organs such as the heart, vasculature, kidneys, gastrointestinal tissue, and peripheral nerves, leading to multiorgan damage.¹ Soluble prefibrillar forms of LC

(monomers and oligomers) play a significant role in tissue injury.^{2,3} Our group previously showed that LC induces endothelial dysfunction in human peripheral and coronary microvessels and causes endothelial cell injury through oxidative and nitrative stress^{4–7} that could be reversed by antioxidant treatment.⁶ Nanoliposomes are artificial vesicles with diameters <100 nm and composed of various phospholipids that were recently shown to have affinity for amyloid proteins, both β -amyloid^{8,9} and LC.¹⁰ They may be the ideal type of nanoparticles for disease treatment because their compositions can be varied to produce desired physiological effects, and they can be loaded with therapeutic cargo; in addition to this structural versatility, they have the advantage of being nonimmunogenic and fully biodegradable and have low cytotoxicity.^{9,11} We recently showed that phosphatidic acid-containing nanoliposomes restored endothelial function in microvessels exposed to LC and preserved endothelial cell viability,⁷ effects that parallel beneficial protection in human microvessels exposed to β -amyloid peptides.¹²

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Monosialogangliosides (GM1 ganglioside) are glycosphingolipids found in plasma membranes and may have a potential advantage over phosphatidic acid in AL because they were found to have antioxidant properties in brain and cardiac tissues^{13–15} and were shown to reduce myocardial ischemia–reperfusion injury,¹⁴ attributes that could be beneficial in reversing LC-induced oxidative stress and cell injury. Our group formulated GM1 ganglioside-containing nanoliposomes (NLGM1), and the aims of this study were to test the hypothesis that NLGM1 protects against LC-induced human endothelial dysfunction and to identify the potential mechanism underlying the protection.

Methods

Human LC Purification

LC from the urine of 4 male participants with biopsy-proven AL with cardiac involvement (aged 51 ± 5.6 years, 2 λ and 2 κ type) were purified using dialysis, size exclusion filtration, and Affi-Gel blue (Bio-Rad) filtration, as described previously.⁶ Protein was verified by Western blot and enzyme-linked immunosorbent assay using antiserum to human λ and κ (Sigma-Aldrich). A fifth LC, AL-09FL, is a recombinant LC derived from a $\kappa 1$ light chain variable domain from a patient with cardiac AL who died 1 year after diagnosis, and the protein sequence was deposited in GenBank (AF490909). The expression vectors for AL-09FL were expressed in Rosetta-gami cells (EMD Millipore, Billerica, MA), as described previously.¹⁶ Briefly, proteins were expressed as inclusion bodies that were solubilized using 8 mol/L urea. Samples were dialyzed against 10 mmol/L Tris-HCl and loaded onto a Superdex 75 column (GE Healthcare). SDS-PAGE gels were run to ensure the purity of the fractions. The patients who provided human-derived LC gave informed consent for collection, and the study was approved by and under the supervision of the institutional review boards of the Phoenix Veterans Affairs Health Care System and the Medical College of Wisconsin.

Nanoliposomes

NLGM1 was prepared from phosphatidylcholine, cholesterol, and GM1 ganglioside (molar ratios 70:25:5) by a lipid film hydration method, similar to previous preparation methods.¹² Briefly, all lipid components were mixed together in chloroform and dried in a rotary vacuum evaporator to completely remove the solvent until a thin lipid film was formed. The lipid film was then hydrated with HEPES solution, pH 7.4, to obtain a final lipid concentration of 10 mg/mL. The resulting liposomal suspension was sonicated for 45 minutes in an ice bath to obtain small unilamellar vesicles or

nanoliposomes. Larger lipid aggregates and titanium particles resulting from sonication were removed by centrifugation at 101 g for 15 minutes at 4°C.

Human Adipose Arteriole Vasoreactivity

Following informed consent from volunteers, subcutaneous abdominal adipose tissues were obtained from 15 male participants (aged 63.6 ± 2.7 years) without AL, known cardiovascular disease, or diabetes mellitus, during planned elective abdominal surgeries for clinical indications (inguinal or umbilical herniorrhaphies). Five patients had hypertension, 1 had hyperlipidemia, and 2 had both hypertension and hyperlipidemia. Of 7 patients with hypertension, 2 were not taking antihypertensive medications, 2 were taking lisinopril, 1 was taking amlodipine, 1 was taking lisinopril/hydrochlorothiazide, and 1 was taking atenolol/chlorthalidone. One patient was on simvastatin. Three were active smokers, and 3 were previous smokers. Arterioles (≈ 80 – 300 μm diameter) were isolated, cannulated, and pressurized to 60 mm Hg (estimated physiological pressure of similarly sized vessels in vivo), similar to previously described methods.⁶ Following precontraction with endothelin 1 (10^{-9} – 10^{-4} mol/L) to achieve $\approx 60\%$ of maximum diameter, baseline control dilator response to acetylcholine (endothelium-dependent dilation, 10^{-9} – 10^{-4} mol/L) and papaverine (smooth muscle-dependent dilation, 10^{-4} mol/L) was measured by videomicroscopy. Following washout, arterioles were then exposed (1 hour) to 20 $\mu\text{g}/\text{mL}$ LC with or without NLGM1 (1:10 mass ratio) and with or without L-NAME (specific nitric oxide synthase [NOS] inhibitor, 5 mmol/L), and a second (posttreatment) dilator response to acetylcholine and papaverine was measured. The dose of LC was chosen because it was within known physiological concentrations of LC in patients.¹⁷ The NLGM1 concentration was chosen because it was similar to the concentration of a phosphatidic acid-containing nanoliposome that conferred a protective effect against β -amyloid and LC.^{7,12}

Endothelial Cell Nitric Oxide Production

Human umbilical vein endothelial cells (HUVECs; passage 4–8; Lonza, Walkersville MD) were seeded evenly into 10-cm² conical culture tubes 24 to 48 hours prior to treatment. Cells were counted immediately before treatment by microscope. Cells were treated with vehicle or LC (20 μg) with or without NLGM1 (1:10 ratio for LC:NLGM1 mass) and with or without L-NAME 5 mmol/L and sealed for 18 to 20 hours. Nitric oxide (NO) head gas readings were measured by piercing the seal and diverting to a calibrated Sievers 280 Nitric Oxide Analyzer (GE Analytical Instruments) and normalized to total cell count. Results are expressed as values relative to vehicle control.

Endothelial Cell Viability and Superoxide, Reactive Oxygen Species, and Peroxynitrite Production

Superoxide production was detected by fluorescent reaction of dihydroethidium¹⁸ (Molecular Probes), whereas peroxynitrite production was detected using coumarin boronic acid pinacolate ester¹⁹ (Cayman Chemical), similar to previous methods.¹² HUVECs were seeded into 12-well plates with glass coverslips in the bottom 16 to 20 hours before undergoing treatment with vehicle control, LC with or without NLGM1, or NLGM1 for 18 to 20 hours. Cells were washed and then stained for 15 minutes at 37°C with 5 μmol/L hydroethidine or 20 μmol/L coumarin boronate in HEPES buffer (in mmol/L: 10 HEPES acid, 138 NaCl, 4 KCl, 1.2 MgSO₄, 1.6 CaCl₂, 1.2 KH₂PO₄, 6 D-glucose, 0.03 EDTA; pH 7.4). After staining, cells were washed again and fixed in 4% paraformaldehyde in PBS, followed by cold methanol and then mounted on glass slides. Slides were imaged on an EVOS FL Auto fluorescent microscope (Life Technologies; Thermo Fisher Scientific) using the RFP light cube (excitation 531/40 nm; emission 593/40 nm) for reacted hydroethidine products or the DAPI light cube (excitation 357/44 nm; emission 447/60 nm) for 7-hydroxycoumarin. Images were measured using ImageJ 1.49 analysis software (National Institutes of Health). To assess reactive oxygen species generation, separate sets of HUVECs underwent the same treatments but were stained with 20 μmol/L of carboxy-2', 7'-dichlorodihydrofluorescein diacetate (Thermo Fisher Scientific) for 15 minutes, followed by washing, fixation, and fluorescent microscopy using the GFP light cube (excitation 470/22 nm; emission 510/42 nm).

In separate experiments, cell viability was assessed by fluorescence of calcein acetoxymethyl, a nonfluorescent compound that can pass through the cell membrane and requires hydrolysis by intact endogenous esterases to release fluorescent anion calcein.²⁰ In brief, cells treated for 18 to 20 hours with vehicle control, LC with or without NLGM1, or NLGM1 were lifted with trypsin into flow cytometry tubes, where they were washed and resuspended in 10 nmol/L calcein acetoxymethyl (Life Technologies; Thermo Fisher Scientific) HEPES buffer containing 1.6 mmol/L calcium. Cells were allowed to stain for 15 minutes at 37°C before washing with HEPES buffer and reading with excitation at 488 nm on the FL-1 channel of Beckman Coulter's FC500 flow cytometer.

Endothelial Cell Gene and Protein Expression

Gene expression was assessed using quantitative polymerase chain reaction. HUVECs were lysed, and the RNA was extracted and converted to cDNA using the Bio-Rad Aurum Total RNA Mini Kit and iScript cDNA synthesis kit (Bio-Rad).

Heme oxygenase 1 (HO-1) and NAD(P)H quinone dehydrogenase 1 (NQO1) are proteins known to protect against oxidative stress, whereas nuclear factor erythroid 2 like 2 (Nrf-2) is a transcription factor that regulates the expression of antioxidant proteins (including HO-1 and NQO1). Primers for HO-1, NQO1, and Nrf-2 were purchased from Integrated DNA Technologies. Polymerization signal was detected using SybrGreen (Bio-Rad) on the Thermal Cycler CFX96 Real-Time System (Bio-Rad) with β-actin primer as the reference gene for normalization.

To determine endothelial NOS (eNOS) protein content, HUVECs treated for 18 to 20 hours with vehicle control, LC with or without NLGM1, or NLGM1 were lysed in a complete radioimmunoprecipitation assay–based lysis buffer containing dithiothreitol, phenylmethanesulfonyl fluoride, and phosphatase inhibitor cocktails (Sigma-Aldrich). Lysates were sonicated, and the DNA was removed by centrifugation. Protein content was determined by Bradford assay, and 30 μg of protein was loaded for electrophoresis in precast Mini-Protean TGX gel (Bio-Rad). Protein was transferred for Western blot to polyvinylidene fluoride low-fluorescence membrane for >1 hour at 100 mV. Antibodies against total eNOS and phosphorylated eNOS (threonine 495 and serine 1177; Cell Signaling) were used at 1:1000 dilution, and all membranes were blocked a second time before developing via 680 or 800CW infrared-fluorescent conjugated goat secondary antibody (LI-COR). Infrared fluorescent bands were detected using the LI-COR Odyssey Cix infrared imaging system, and signal was reported as measured using Image Studio 4.0 (LI-COR) normalized to β-actin, which was detected in the same manner. For detecting protein expression of HO-1 and NQO1, proteins were blotted in a manner similar to that described for eNOS expression. HO-1 and NQO-1 antibodies were purchased from Cell Signaling. To assess translocation of Nrf-2 protein in the nuclear region, separate sets of HUVECs were treated with vehicle, LC with or without NLGM1, or NLGM1 for 18 to 20 hours. Following treatment, cells were trypsinized and collected on ice. Nuclear proteins were separated using nuclear and cytoplasmic extraction reagents, according to the manufacturer's protocol (Thermo Fisher Scientific). Nuclear Nrf-2 proteins were assessed by Western blotting of isolated nuclear proteins, as described previously, and the signal was measured and normalized to loading control protein tubulin; cytosolic Nrf-2 proteins were also measured and normalized to loading control protein actin.

Gene Transfection Experiments

HUVECs were subjected to RNA interference at passage 4 to 6 in 6-well plates with ≈75% to 85% confluence. Three predesigned siRNAs against Nrf-2 and control siRNAs

(Integrated DNA Technologies) were transfected into HUVECs using HiPerFect (Qiagen) transfection reagent, according to the manufacturers' recommendations. The nucleotide sequences of the Nrf-2 siRNAs are as follows: (S1) 5'-ACCUGUCUCUUAUCUAGUUGUAACUG, (S2) 5'-AUCUUUCAAUGAUCUAAAUCUUGCUC, (S3) 5'-UGCUCUUUGGACAUCAUUUCGUUGAAG. Lipid-transfection complexes were made with 300 ng (100 ng each) of Nrf-2 siRNAs and either 6 μ L (low) or 12 μ L (high) of HiPerFect reagent to ensure adequate knockdown. Control complexes consisted of 300 ng of control siRNAs and 12 μ L HiPerFect. Completeness of Nrf-2 knockdown was evaluated by comparing control siRNAs with 12 μ L HiPerFect to Nrf-2 siRNAs with both 6 and 12 μ L of transfection reagent. The following day, cells were washed once in 1 \times PBS and given fresh growth media for 6 hours prior to addition of NLGM1 for 24 hours. Because there was no significant difference in outcomes between low and high HiPerFect reagent Nrf-2 siRNA treatments, the results of both treatments were combined. Following verification that Nrf-2 siRNA successfully knocked down Nrf-2, additional HUVECs subjected to control siRNA were exposed to vehicle or LC with or without NLGM1 (18–20 hours), and peroxynitrite production and cell viability were measured, as described previously; the results were then compared with those of HUVECs subjected to Nrf-2 siRNA and exposed to LC with NLGM1.

Data and Statistical Analyses

Data are expressed as mean \pm SEM, and a significant *P* value was set at *P*<0.05 (2-sided). Vasoreactivity was assessed by comparing control (baseline) and posttreatment responses in the same arteriole to maximum acetylcholine dose (10^{-4} mol/L) and papaverine (10^{-4} mol/L), using a paired Student *t* test, whereas responses between the 2 treatments were analyzed using an unpaired Student *t* test. To test for overall acetylcholine curve effect (response to doses of 10^{-9} – 10^{-4} mol/L), we compared the half-maximal effective concentration, the dose of acetylcholine that produced 50% maximum dilation, similar to our published method.⁶ In brief, half-maximal effective concentration was derived using nonlinear regression with a variable slope (4 parameters) and least squares ordinary fit (GraphPad Prism 5.0; GraphPad Software). For endothelial cell assays, group (treatment) comparisons were evaluated using 1-way repeated-measures ANOVA with post hoc pairwise testing using the Holm–Sidak method or Tukey test for normally distributed data and 1-way repeated-measures ANOVA on ranks with post hoc pairwise testing using Dunn's method for data sets that do not pass a normality test. For data sets with unequal sample sizes and nonnormal data distribution, 1-way ANOVA on ranks was done and post hoc pairwise testing was performed using Dunn's method. All post hoc pairwise comparisons were based on

multiple comparison procedures. Statistical analyses were performed using SigmaStat 3.5 (SigmaStat) and GraphPad Prism 5.0.

Results

Human Arteriole Vasoreactivity

As shown in Figure 1A and 1B, LC caused significant reduction in dilation response to acetylcholine, signifying impaired endothelium-dependent dilation; this was restored by cotreatment with NLGM1. NLGM1 alone did not affect dilator response to acetylcholine. Cotreatment with L-NAME abolished the protective response of NLGM1 to LC treatment. LC did not cause significant reduction in dilator response to papaverine versus control (*P*=0.07), signifying no significant change in smooth muscle–dependent dilation.

Endothelial Cell NO Production and eNOS Protein Expression

LC caused significant reduction in NO production after 18 to 20 hours of exposure (Figure 2A). Cotreatment with NLGM1 restored NO production, whereas NLGM1 alone showed no significant difference from control. NOS inhibitor L-NAME abolished the protective effect of NLGM1 on LC-treated cells on NO production. There was no significant difference in total and phosphorylated eNOS protein expression and phosphorylated eNOS (threonine 495)/eNOS ratio among endothelial cells treated with vehicle, LC, LC with NLGM1, and NLGM1 (Figure 2B). Similar results were observed with phosphorylated eNOS (serine 1177); the phosphorylated eNOS/eNOS ratio among the groups was also not significantly different (Figure 2C).

Endothelial Cell Superoxide, Peroxynitrite Production, and Cell Viability

Exposure to LC caused increased production of superoxide and peroxynitrite (Figure 3A and 3B). NLGM1 cotreatment decreased LC-induced superoxide and peroxynitrite production. Reactive oxygen species production using dichlorodihydrofluorescein fluorescence was also measured. LC increased reactive oxygen species production, and cotreatment with NLGM1 decreased LC-induced increased reactive oxygen species (control 1 ± 0 , LC 1.91 ± 0.2 , LC with NLGM1 1.38 ± 0.12 , NLGM1 1.24 ± 0.21 , overall ANOVA *P*=0.03, *P*<0.05 LC versus control and *P*<0.05 LC versus LC with NLGM1). Exposure to LC resulted in significant reduction in the viability of endothelial cells measured by calcein acetoxymethyl fluorescence (Figure 3C). Cotreatment with NLGM1 restored HUVEC viability; NLGM1 alone did not affect cell viability.

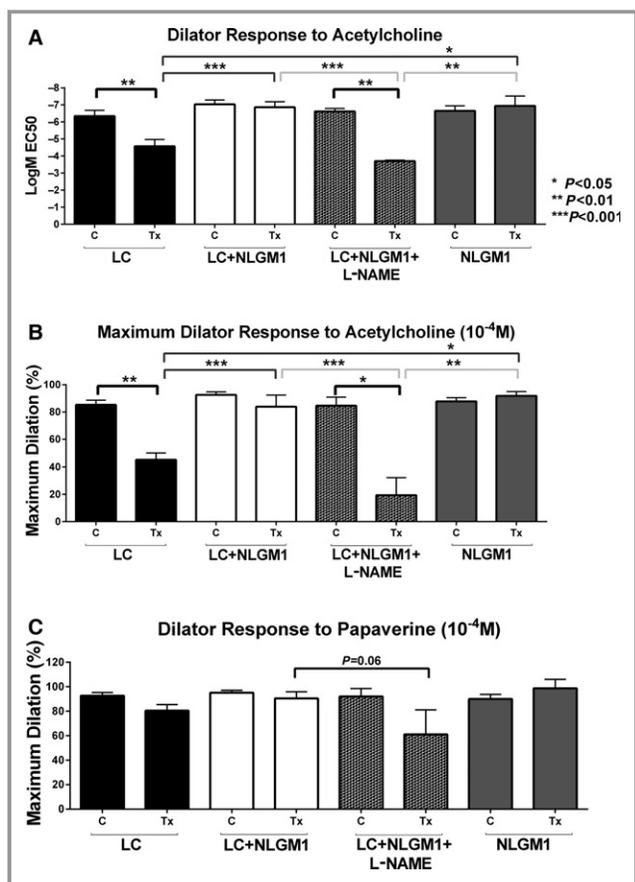


Figure 1. Arteriole vasoreactivity. A, EC50 dose of acetylcholine (log mol/L; note that the *y*-axis scale is inverted). B, Maximum dilator response to acetylcholine (10⁻⁴ mol/L). Treatments were compared with baseline control (paired analyses) and with other treatments (unpaired analyses). Exposure to LC resulted in reduced dilator response to acetylcholine compared with baseline control response (n=12). Cotreatment of LC with monosialoganglioside-containing nanoliposomes (NLGM1) fully restored dilator response to acetylcholine (n=14). The NLGM1-protective response in LC-treated arterioles was abolished by cotreatment of L-NAME, a specific inhibitor of nitric oxide synthase (LC with NLGM1 and L-NAME plot, n=3). Dilator response to NLGM1 alone did not differ from baseline control (n=3). C, Dilator response to papaverine. There was no significant change in dilator response to papaverine with treatment with LC, LC with NLGM1, LC with NLGM1 and L-NAME, or NLGM1 alone. Dilator response to papaverine did not differ significantly among the various treatments. C indicates control; EC50, half-maximal effective concentration; LC, light chain proteins; NLGM1, GM1 ganglioside-containing nanoliposomes; Tx, treatment.

Endothelial Cell Stress Response (HO-1, NQO1, and Nrf-2)

Exposure to LC for 18 to 20 hours did not change HO-1 or NQO1 gene or protein expression (Figure 4). NLGM1 cotreatment with LC showed significant increases in HO-1 and NQO1 gene and protein expression compared with LC-treated cells

or controls. By itself, NLGM1 caused increased HO-1 and NQO1 gene and protein expression versus control.

The Nrf-2 protein content in the nucleus of endothelial cells was increased in cells treated with NLGM1 compared with control (Figure 5A). Light chains did not increase nuclear Nrf-2 versus control, but cotreatment of LC with NLGM1 showed a significant increase in Nrf-2 compared with LC-treated cells alone. Cytosolic Nrf-2 protein contents were as follows: control 1±0, LC 0.64±0.11, LC with NLGM1 1.28±0.25, NLGM1 1.62±0.40 (ANOVA *P*=0.008, n=8). Pairwise comparison showed that cytosolic Nrf-2 protein in cells treated with NLGM1 was significantly higher than in LC-treated cells (*P*<0.05), but the rest of the pairwise comparisons were not significantly different. To assess whether the increase in HO-1 and NQO1 gene expression by NLGM1 is mediated by Nrf-2 signaling, HUVECs were transfected with Nrf-2 siRNA. There was a significant reduction in Nrf-2 gene expression in HUVECs transfected with Nrf-2 siRNA versus control siRNA, showing effective downregulation of Nrf-2 gene expression (Figure 5B). HUVECs transfected with control siRNA showed significant increases in HO-1 and NQO1 gene expression when exposed to NLGM1 (Figure 5C and 5D); this increase in gene expression was abolished in HUVECs transfected with Nrf-2 siRNA. To evaluate whether suppression of Nrf-2 gene expression reversed the protective effects of NLGM1, HUVECs were transfected with either control or Nrf-2 siRNA. HUVECs treated with control siRNA and exposed to LC showed increased peroxynitrite and reduced cell viability that were reversed by NLGM1 cotreatment (Figure 5E and 5F). The protective effects of NLGM1 against LC were abolished in HUVECs treated with Nrf-2 siRNA.

Discussion

Our results revealed the following novel and important findings. First, NLGM1 reverses LC-induced human arteriole endothelial dysfunction and preserves viability of endothelial cells exposed to LC. Second, the mechanism of protection by NLGM1 involves reduction of oxidative and nitrative stress through induction of antioxidant stress cellular protective mechanisms mediated through Nrf-2 signaling. These findings point to a potential novel therapeutic approach against LC-induced cellular injury in AL.

AL is a disease that arises from overproduction by plasma cells of amyloidogenic LC that misfolds and deposits in various organs as amyloid, causing multiorgan damage.¹ Much evidence suggests that tissue toxicity derives, in large part, from the soluble prefibrillar forms of LC that induce oxidative and nitrative stress.^{2,3,5,6,21–23} Like other amyloid diseases such as Alzheimer's disease, vascular involvement (both coronary and peripheral) appears to be an early and prominent pathology in AL.^{24,25} We showed that acute

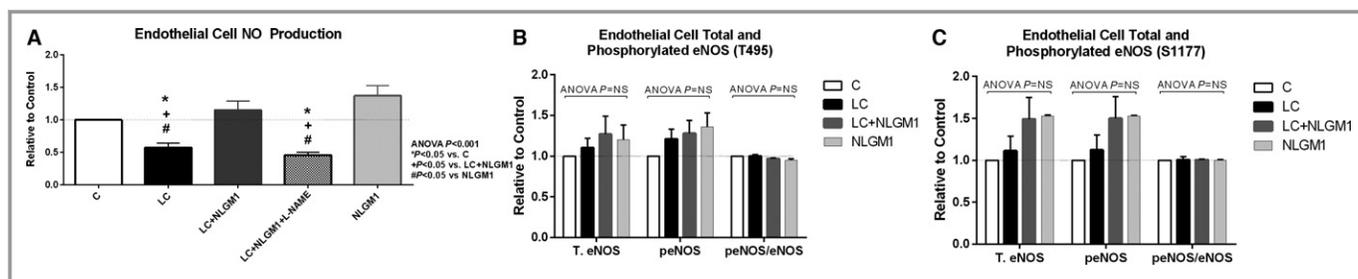


Figure 2. Endothelial cell NO production and eNOS expression. A, Human umbilical vein endothelial cells exposed to 18 to 20 hours of LC showed significant reduction in NO head gas production compared with control. Cotreatment with NLGM1 restored NO production. There was no difference in NO production between endothelial cells treated with NLGM1 and vehicle control ($n=10$ for C, LC, LC with NLGM1, $n=7$ for LC with NLGM1 and L-NAME, and $n=6$ for NLGM1). B, Protein expression of total eNOS and peNOS (threonine 495) and phosphorylated/total eNOS ratios were not significantly different among cells treated with vehicle, LC, LC with NLGM1, or NLGM1 ($n=7$). C, Similar results were observed with peNOS (serine 1177) ($n=4$). C indicates control; eNOS, endothelial nitric oxide synthase; LC, light chain proteins; NLGM1, GM1 ganglioside-containing nanoliposomes; NO, nitric oxide; NS, not significant; peNOS, phosphorylated endothelial nitric oxide synthase; T, total.

exposure to physiological doses of LC purified from participants with AL resulted in endothelial dysfunction, oxidative stress, reduced NO bioavailability, and endothelial cell death.^{5,6} Using selective antioxidant (mitoquinone), selective inhibitor (gp91ds-tat) and eNOS cofactor supplementation (tetrahydrobiopterin), we previously showed that increased superoxide production by endothelial cells following exposure to LC are likely from multiple sources including mitochondria, NADPH oxidase, and eNOS uncoupling.⁶ Untreated AL is associated with poor prognosis, with median survival of 4 months in the setting of cardiac failure.²⁶ Chemotherapy augmented by autologous stem cell transplantation is the treatment of choice, but this approach is associated with high treatment-related mortality; therefore, the sickest patients, such as those with advanced heart failure, are often ineligible for this treatment or are limited to less aggressive regimens.^{27,28} In addition, despite widespread use of chemotherapy, significant morbidity and mortality remain.^{29–32}

Nanoliposomes are artificial phospholipid vesicles <100 nm diameter that may be useful for amyloid diseases.

Unlike other nanoparticles, they are nonimmunogenic, fully degradable, and structurally versatile and have lower cytotoxicity.^{9,11} Nanoliposomes containing cholesterol and phosphatidic acid were shown to bind to β -amyloid peptides^{8,9} and to alter the secondary structure of LC, as seen on circular dichroism spectroscopy, while reducing LC internalization in endothelial cells.⁷ These effects on LC were associated with preservation of endothelial function and protection against LC-induced endothelial cell death.⁷

Our results show that NLGM1 restores human microvascular endothelial function impaired by LC. The lack of difference in vascular response between NLGM1 alone and baseline control suggests that the effect of NLGM1 does not occur through an intrinsic vasodilator effect. Abolition of NLGM1 protection with the specific NOS inhibitor L-NAME in arterioles and reversal of LC-induced reduction of NO in endothelial cells suggest that the vascular protection is mediated through increased NO bioavailability. LC causes increased endothelial cell superoxide and peroxynitrite production and reduced NO production without altering eNOS,

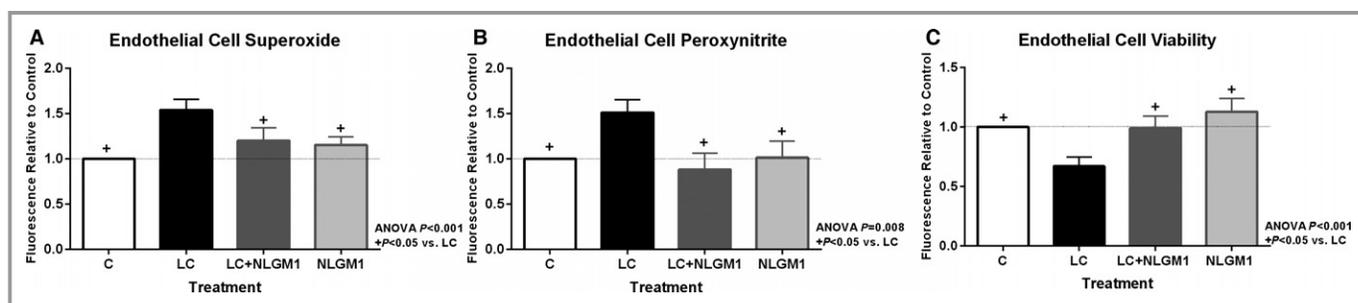


Figure 3. Endothelial cell superoxide, peroxynitrite production, and viability. A, HUVECs treated with LC showed increased superoxide production that was reversed by cotreatment with NLGM1 ($n=17$). B, There was increased peroxynitrite in LC-treated cells; this increase was abolished by cotreatment with NLGM1. NLGM1 alone showed no difference in peroxynitrite production compared with vehicle ($n=11$). C, Cell viability assessed using calcein acetoxyethyl fluorescence showed reduced HUVEC viability following treatment with LC. Cell viability was restored by NLGM1 cotreatment ($n=14$). C indicates control; HUVEC, human umbilical vein endothelial cell; LC, light chain proteins; NLGM1, GM1 ganglioside-containing nanoliposomes.

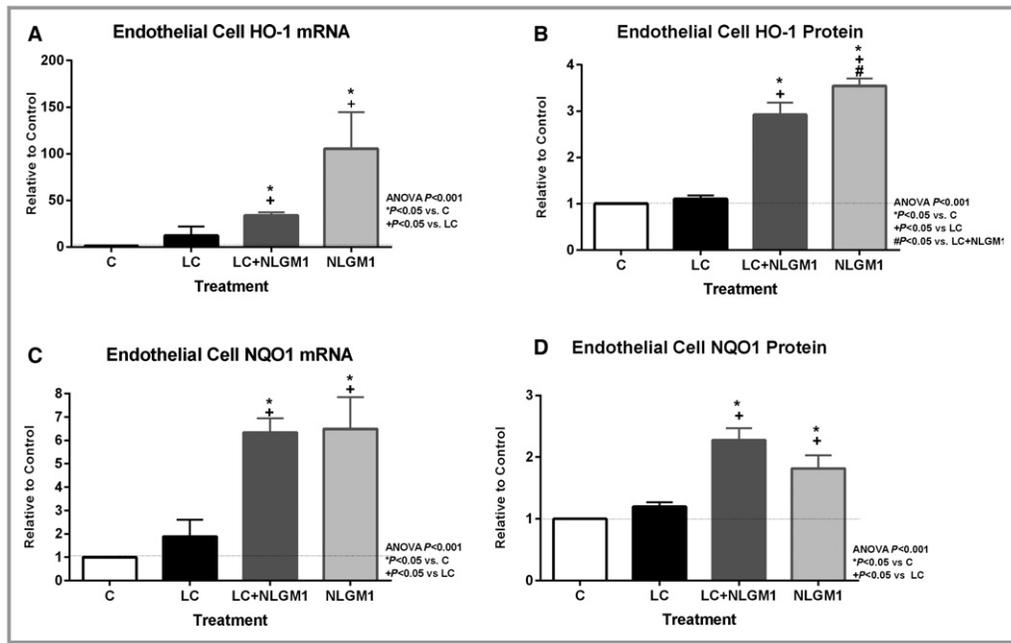


Figure 4. Endothelial cell gene and protein expression. A, Human umbilical vein endothelial cells treated with LC showed no significant increase in HO-1 gene expression compared with control. Cotreatment with NLGM1 and NLGM1 alone resulted in significant increases in HO-1 gene expression compared with control or LC-treated cells ($n=13$ control, $n=11$ LC, $n=17$ LC plus NLGM1, and $n=11$ NLGM1). B, A similar pattern was seen for protein expression of HO-1 except there was a significant difference in HO-1 between cells treated with NLGM1 and with LC with NLGM1 ($n=7$ each treatment). C, NLGM1 increased NQO1 gene expression when given to cells alone or as cotreatment with LC compared with control or LC-treated cells ($n=12$ control, $n=11$ LC, $n=17$ LC with NLGM1, $n=10$ NLGM1). D, NLGM1 also increased NQO1 protein expression when given to cells alone or as cotreatment with LC compared with control or LC-treated cells ($n=8$). C indicates control; HO-1, heme oxygenase 1; LC, light chain proteins; NLGM1, GM1 ganglioside-containing nanoliposomes; NQO1, NAD(P)H quinone dehydrogenase 1.

phosphorylated eNOS, and the phosphorylated eNOS/eNOS ratio, suggesting that eNOS uncoupling may be a mechanism of reduced NO bioavailability and increased oxidative and nitrative stress; however, other sources of increased superoxide may also be involved, as we showed previously.⁶ The current findings are congruent with our previous finding that an agent that restored eNOS coupling, the cofactor tetrahydrobiopterin, reversed LC-induced microvascular endothelial dysfunction.⁶ NLGM1 cotreatment reduced superoxide and peroxynitrite and increased NO production in LC-treated endothelial cells; the reduced oxidative and nitrative stress likely plays a role in protecting endothelial cells against LC-induced cell toxicity, as measured by calcein acetyloxymethyl fluorescence. Peroxynitrite is a reactive nitrogen species formed by the reaction of NO and superoxide and is one of the most potent mediators of DNA and protein damage.³³

Our results showed that the protective effects of NLGM1 arise, at least in part, from a direct effect induced by NLGM1 in initiating endothelial cell antioxidant protective stress response mechanisms. NLGM1 increased gene and protein expression of HO-1 and NQO1. HO-1 is the inducible isoform

of an enzyme that functions as a defense mechanism against oxidative stress through cleavage of heme that yields biliverdin or bilirubin, known physiological antioxidants, in addition to carbon monoxide, a physiological activator of guanylyl cyclase like NO.³⁴ NQO1 is a highly inducible flavin adenine dinucleotide-dependent flavoprotein enzyme that has antioxidant effects through promotion of 2-electron reductions of quinones and depression of quinone levels that lead to reduced generation of reactive oxygen intermediates by redox cycling.³⁵ Both HO-1 and NQO1 expression are regulated by Nrf-2, a transcription factor that regulates the expression of antioxidant proteins to protect against oxidative damage triggered by injury and inflammation.³⁶ Our results showed that NLGM1 caused an increase in endothelial cell Nrf-2 protein in the nucleus that most likely led to the increased gene and protein expression of both HO-1 and NQO1; the increased gene expression of HO-1 and NQO1 induced by NLGM1 was abolished when Nrf-2 gene expression was suppressed. The causal link between induction by NLGM1 of Nrf-2/antioxidant stress response and protection against LC injury is supported by our finding that Nrf-2

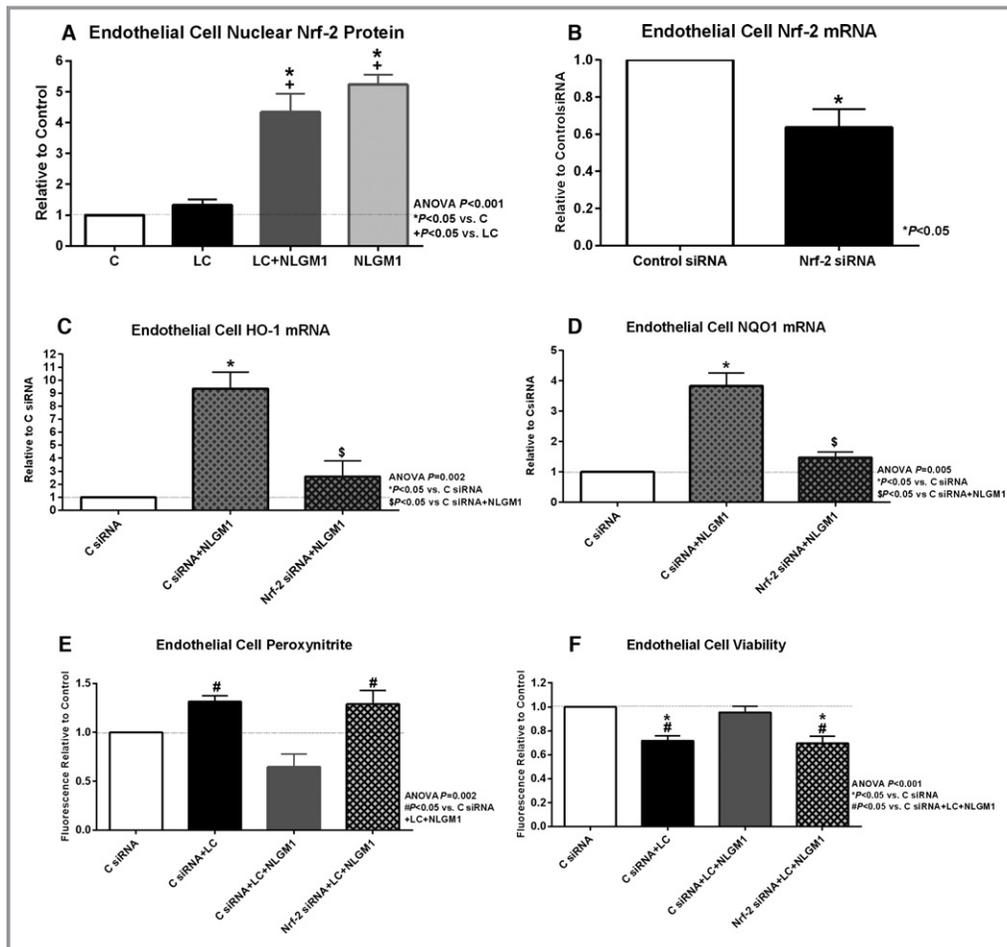


Figure 5. Endothelial cell Nrf-2. A, Treatment with NLGM1 or cotreatment of LC with NLGM1 resulted in increased nuclear Nrf-2 protein compared with vehicle control or LC-treated cells (n=8). B through E, Endothelial cell Nrf-2 siRNA transfection. B, There is reduced Nrf-2 gene expression in HUVECs transfected with Nrf-2 siRNA compared with control siRNA, showing effective suppression of gene expression (n=6). C and D, NLGM1 increased HO-1 and NQO1 gene expression in HUVECs transfected with control siRNA; this increase was abolished in HUVECs transfected with Nrf-2 siRNA (n=3). E and F, In HUVECs treated with control siRNA, LC increased peroxynitrite (n=5) and reduced cell viability (n=6); these effects were reversed by cotreatment with NLGM1. The protective effect of NLGM1 was reversed in HUVECs treated with Nrf-2 siRNA. C indicates control; HO-1, heme oxygenase 1; HUVEC, human umbilical vein endothelial cell; LC, light chain proteins; NLGM1, GM1 ganglioside-containing nanoliposomes; NQO1, NAD(P)H quinone dehydrogenase 1; Nrf-2, nuclear factor erythroid 2 like 2.

knockdown using siRNA abrogated NLGM1 protective effects. Why NLGM1 triggers this antioxidant response is not known and would be a focus of future investigation.

Our results are novel and distinct from our previous publication,⁷ especially in 2 important respects. First, we purposefully changed the nanoliposome composition to include GM1 ganglioside. The rationale for this choice was that, unlike phosphatidic acid, GM1 ganglioside was shown to have antioxidant properties in neural tissue exposed to ischemia or toxic agents such as glutaric acid through inhibition of lipid peroxidation or free radical scavenging.^{13,15,37,38} Furthermore, rat hearts pretreated with gangliosides exposed to ischemia–reperfusion were also shown to

have reduced lipid peroxidation, hydroxyl radical formation, and better myocardial preservation.¹⁴ Second, we report for the first time that the mechanism, or one of the mechanisms, by which NLGM1 protects against LC endothelial injury is through Nrf-2–mediated upregulation of antioxidant stress responses (HO-1 and NQO1), leading to reduced oxidative and nitrate stress and increased NO bioavailability. Because we previously demonstrated that oxidative and nitrate stress underlie microvascular injury induced by amyloid proteins (LC in AL,⁶ β -amyloid in Alzheimer’s disease,³⁹ and medin in aortic medial amyloidosis⁴⁰), the induction of an antioxidant stress response by NLGM1 may have potential clinical relevance not only to AL but also to other amyloid diseases.

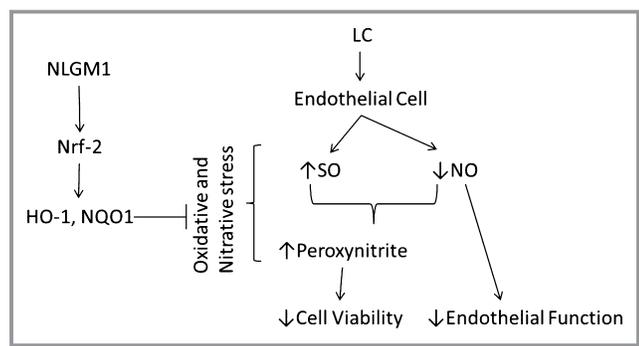


Figure 6. Proposed schema by which NLGM1 confers protection against LC-induced endothelial dysfunction. LC causes reduced endothelial cell NO production that leads to endothelial dysfunction. LC also causes increased oxidative and nitrative stress that leads to reduced endothelial cell viability. NLGM1 induces antioxidant stress responses (Nrf-2, HO-1 and NQO1) that lead to reduced nitrative stress, increased NO bioavailability, increased endothelial cell viability, and restoration of endothelial function. HO-1, heme oxygenase 1; LC, light chain proteins; NLGM1, GM1 ganglioside-containing nanoliposomes; NO, nitric oxide; NQO1, NAD(P)H quinone dehydrogenase 1; Nrf-2, nuclear factor erythroid 2 like 2; SO, superoxide.

Our study has several important limitations. AL-induced vascular dysfunction affects both peripheral and central (coronary) arterioles, and we tested efficacy only on peripheral adipose arterioles; however, we previously demonstrated similarity in response to LC between adipose and coronary arterioles,⁶ validating the adipose arteriole model as a reasonable surrogate for coronary arteriole response. Similarly, vasomotor responses in ex vivo arterioles paralleled the functional dysfunction observed in brachial artery flow-mediated dilation in living participants,⁴¹ suggesting that our ex vivo model recapitulates in vivo treatment responses. Our arteriole model is limited in testing only acute effects and not chronic exposure, so preclinical testing in animal models will be necessary to determine whether the protective effects of NLGM1 are durable and replicable in vivo. In addition, although we used a primary human endothelial cell line (HUVEC), the degree of phenotypic similarity with endothelial cells in intact human arterioles is not fully established. The effects of LC on larger conduit arteries were not tested and need to be studied in the future. Although we did not test for sources of superoxide or reactive oxygen species in this study, we previously showed that the increased endothelial cell superoxide production induced by LC was likely from multiple sources (mitochondria, NADPH oxidase, and eNOS uncoupling).⁶

In summary, we found that light chains induce human adipose arteriole endothelial dysfunction, reduced NO bioavailability, increased superoxide and peroxynitrite and reduced endothelial cell viability. NLGM1 cotreatment restored endothelial function and cell viability by increasing NO bioavailability, reducing peroxynitrite production and

promoting antioxidant response through a Nrf-2-mediated signaling mechanism, as summarized in Figure 6. NLGM1 is a potential novel treatment approach for AL that deserves further investigation and therapeutic development.

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Disclosures

None.

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