Human microvascular dysfunction and apoptotic injury induced by AL amyloidosis light chain proteins

Raymond Q. Migrino,1,2 Seth Truran,1,2 David D. Gutterman,2 Daniel A. Franco,1 Megan Bright,2 Brittany Schlundt,2 Mitchell Timmons,2 Angelica Motta,1 Shane A. Phillips,2 and Parameswaran Hari2

1Department of Cardiology and Office of Research, Phoenix Veterans Affairs Health Care System, 2Department of Medicine, Medical College of Wisconsin, Milwaukee, Wisconsin

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Migrino RQ, Truran S, Gutterman DD, Franco DA, Bright M, Schlundt B, Timmons M, Motta A, Phillips SA, Hari P. Human microvascular dysfunction and apoptotic injury induced by AL amyloidosis light chain proteins. Am J Physiol Heart Circ Physiol 301: H2305–H2312, 2011. First published September 30, 2011; doi:10.1152/ajpheart.00503.2011.—Light chain amyloidosis (AL) involves overproduction of amyloidogenic light chain proteins (LC) leading to heart failure, yet the mechanisms underlying tissue toxicity remain unknown. We hypothesized that LC induces endothelial dysfunction in non-AL human microvasculature and apoptotic injury in human coronary artery endothelial cells (HCAECs). Adipose arterioles (n = 34, 50 ± 3 yr) and atrial coronary arterioles (n = 19, 68 ± 2 yr) from non-AL subjects were cannulated. Adipose arteriole dilator responses to acetylcholine/papaverine were measured at baseline and 1 h exposure to LC (20 μg/ml) from biopsy-proven AL subjects (57 ± 11 yr) without and with antioxidant cotreatment. Coronary arteriole dilation to bradykinin/ papaverine was measured post-LC exposure. HCAECs were exposed to 1 or 24 h of LC. LC reduced dilation to acetylcholine (10−4 M; 41.6 ± 7 vs. 85.5 ± 2.2% control, P < 0.001) and papaverine (81.4 ± 4.6 vs. 94.8 ± 1.3% control, P < 0.01) in adipose arterioles and to bradykinin (10−6 M; 68.6 ± 6.2 vs. 90.9 ± 1.6% control, P < 0.001) but not papaverine in coronary arterioles. There was an increase in superoxide and peroxynitrite in arterioles treated with LC. Adipose arteriole dilation was restored by cotreatment with polyethylene glycol-superoxide dismutase and tetrahydrobiopterin but only partially restored by mitoquinone (mitochondria-targeted antioxidant) and gp91ds-tat (NADPH oxidase inhibitor). HCAECs exposed to LC showed reduced NO and increased reactive oxygen species (ROS) production, impaired contractility, and apoptotic injury (8, 19). In AL patients, plasma LC level is one of the strongest predictors of mortality (10), and clearance of circulating LC by chemotherapy was associated with resolution of heart failure (11) and nephrotic syndrome (17). There is increasing recognition of the important role of early microvascular dysfunction in the pathophysiology of amyloid diseases. In Alzheimer’s disease, Aβ-amyloid protein-induced cerebral microvascular dysfunction is now recognized as an important contributor to Alzheimer’s pathophysiology (1). In AL amyloidosis, early endothelial dysfunction (impaired brachial and cutaneous flow-mediated dilation) was reported before development of amyloid neuropathy (6). AL amyloid patients were shown to have abnormal brachial artery flow-mediated dilation compared with normal controls (24). Despite absence of epicardial coronary obstruction, 74% of patients with cardiac AL amyloidosis demonstrated histological evidence of myocardial ischemia associated with perivascular amyloid deposition (27). These studies suggest the potential and important role of microvascular dysfunction in the pathophysiology of LC injury. The aims of this study are to (1) test the hypotheses that acute exposure to AL LC will result in endothelial dysfunction in isolated (ex vivo) human adipose and coronary arterioles from subjects without AL and induce apoptotic injury in human coronary artery endothelial cells (HCAEC) and (2) explore potential mechanisms underlying LC-induced microvascular dysfunction and injury.

MATERIALS AND METHODS

Human Subjects

Seven subjects (2 females, 57 ± 4 yr) with biopsy-proven AL (4 lambda, 3 kappa type) provided informed consent and urine for protein collection before initiation of chemotherapy. Organ involvement was as follows: seven cardiac, three gastrointestinal tract, and one renal. The study was approved by local Institutional Review Boards.

Abdominal or thoracic adipose tissues from 34 patients (10 females, age 50 ± 3 yr) undergoing routine abdominal or thoracic surgery were collected. Similarly, discarded atrial tissues from 19 subjects (9 females, age 68 ± 2 yr) undergoing open heart surgery for valvular disease without significant epicardial coronary disease were collected. Subjects with known coronary or peripheral arterial disease, diabetes, systemic infection, or amyloidosis were excluded. The discarded and deidentified tissues were collected under waiver of informed consent (Medical College of Wisconsin) or after obtaining informed consent (Phoenix Veterans Affairs Health Care System) as approved by the local Institutional Review Boards.
Isolation of LC

The LC were purified from the urine of AL patients using dialysis, size exclusion filtration (10, 50-µm Amikon filters), Affigel blue filtration to remove albumin, and lyophilization similar to previous methods (22). Following purification, the proteins were verified to be kappa or lambda by Western blotting using human antibody to kappa or lambda light chains (Sigma-Aldrich, St. Louis, MO). In some adipose experiments, the following control proteins were also used: 1) purified kappa and lambda LC derived from patients with multiple myeloma/Bence Jones proteins without amyloidosis (Genway Biotech, San Diego, CA, and Sigma-Aldrich) and 2) kappa and lambda LC from AL patients subjected to thermal denaturation at 140°C for 1 h (4).

Arteriole Vasodilator Response

Coronary and adipose arterioles were isolated, cannulated, and pressurized to 60 mmHg pressure (estimated physiological pressure of similar-sized vessels in vivo) without flow, similar to previous methods (22, 30). The vessels were preconstricted to ~60% of maximum diameter using endothelin-1 (10⁻⁶ to 10⁻⁴ M). With the use of a videomicroscope (VIA-100 video micrometer; Boeckeler, Tucson, AZ), the dilator response (vessel diameter) was measured at baseline and videomicroscope (VIA-100 video micrometer; Boeckeler, Tucson, AZ), the dilator response was tested again.

Arteriole Fluorescence Microscopy

In separate arterioles, 20 µg/ml LC or vehicle was administered for 1 h. Fluorescence microscopy was used to determine the production of superoxide using hydroethidine (7) (5 µM, 490 nM excitation, 626 nM emission; Invitrogen, Eugene, OR) similar to previous methods (22). In the presence of superoxide, hydroethidine is oxidized to fluorescent hydroxyethidium (30). Whole vessel fluorescence was measured using an Olympus IX51 fluorescence microscope with images recorded with the same computer-specified gain and intensity settings and with correction for background signal. Quantification was performed using Image J 1.44 software, and values were expressed relative to vehicle control. Specificity for superoxide was determined by looking at PEG- and SOD-quenchable fluorescence. Peroxynitrite was assessed using dihydrodorhodamine-123 (5 µM, 488 nM excitation, 525 nM emission; Invitrogen) whose interaction leads to the formation of the fluorescent chemical rhodamine (15). Dynamic nitric oxide (NO) production was assessed using 4,5-diaminofluorescein diacetate (5 µM, 480 nM excitation, 510 nM emission; Calbiochem, Gibbstown, NJ) by measuring fluorescence at baseline and at timed intervals (up to 30 min) following exposure to LC.

Human Coronary Artery Endothelial Cells

HCAEC (LONZA) were grown in EGM-2 MV media (LONZA) with 5% FBS to passages 7 through 9, at which time they were plated to 25-mm solid-capped culture flasks (Griener) and allowed to grow to 100% confluence. Cells were then treated with LC at 20 µg/ml, and the caps were sealed tightly for 24 h to contain the head gas for NO analysis similar to previous methods (13).

Nitric oxide. The head gas was then drawn into a 30-ml syringe and diverted in an air-tight system to a calibrated Sievers 280 NO analyzer (General Electric, Waukesha, WI). Peak NO readings were recorded across three replicates and averaged. The analyzer was allowed to rezero between each reading. Cells were then allowed to equilibrate in a sterile environment before lifting with trypsin and counted with a Coulter Z1 particle counter (Beckmann Coulter, Brea, CA). NO concentration readings were then normalized to cell content (per one million cells). After NO analysis, cells were washed, stained, and fixed for flow cytometry.

Flow cytometry. HCAEC were suspended in binding buffer (HEPES buffer 10 mM/NaOH, pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM MgCl₂, and 1.8 mM CaCl₂). Cells were then incubated for 15 min at room temperature with 0.5 µg/ml annexin V-FITC conjugate (eBiosciences, San Diego, CA) and 0.15 µM propidium iodide (Sigma-Aldrich). Cells were then diluted to 1–2 × 10⁶ cells/ml and fixed with 1% paraformaldehyde. Ten thousand events were run on the flow cytometer (Cytomics Flow Cytometer 500; Beckman Coulter) with excitation 488 and emission 525 (FITC) and 620 (propidium iodide).

Western blot. Endothelial nitric acid (eNOS) and phosphorylated eNOS (phospho-eNOS) levels were measured by Western blot using primary antibody to human eNOS or phospho-eNOS (Abcam, Cambridge, MA). Briefly, HCAEC were lysed in radioimmunoprecipitation buffer (Sigma-Aldrich) and 1 mM dithiothreitol, sonicated, and the DNA pelleted. Protein content was measured using reducing reagent-compatible bicinchoninic acid protein assay (Pierce, Rockford, IL), and lanes were loaded with 30 µg of protein. Primary antibody to human eNOS was used at 1:1,000 and detected via chemiluminescence of horseradish peroxidase-conjugated secondary. Blots were read with the AlphaInnotech FluorChemQ gel sorting unit, and band density was measured using AlphaView for analysis.

Fluorescence microscopy. Light-protected HCAEC were incubated for 60 min at 37°C with 5 µM hydroethidine or 5 µM dihydrodorhodamine. Cells were then suspended at 1–2 × 10⁶ cells/ml and fixed with 1% paraformaldehyde. Fluorescence microscopy measurements were performed similar to procedures outlined for arterioles.

Data and Statistical Analyses

Data are expressed as means ± SE. For vasoreactivity measurements, Student’s t-tests (paired or unpaired) were used to compare dilator responses. To test for overall curve effect, we compared effective concentration 50% (EC₅₀) but this time following intraluminal exposure to LC [20 µg/ml LC or vehicle was administered for 1 h. Fluorescence microscopy was used to determine the production of superoxide using hydroethidine (7) (5 µM, 490 nM excitation, 626 nM emission; Invitrogen, Eugene, OR) similar to previous methods (22). In the presence of superoxide, hydroethidine is oxidized to fluorescent hydroxyethidium (30). Whole vessel fluorescence was measured using an Olympus IX51 fluorescence microscope with images recorded with the same computer-specified gain and intensity settings and with correction for background signal. Quantification was performed using Image J 1.44 software, and values were expressed relative to vehicle control. Specificity for superoxide was determined by looking at PEG- and SOD-quenchable fluorescence. Peroxynitrite was assessed using dihydrodorhodamine-123 (5 µM, 488 nM excitation, 525 nM emission; Invitrogen) whose interaction leads to the formation of the fluorescent chemical rhodamine (15). Dynamic nitric oxide (NO) production was assessed using 4,5-diaminofluorescein diacetate (5 µM, 480 nM excitation, 510 nM emission; Calbiochem, Gibbstown, NJ) by measuring fluorescence at baseline and at timed intervals (up to 30 min) following exposure to LC.

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Flow cytometry. HCAEC were suspended in binding buffer (HEPES buffer 10 mM/NaOH, pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM MgCl₂, and 1.8 mM CaCl₂). Cells were then incubated for 15 min at room temperature with 0.5 µg/ml annexin V-FITC conjugate (eBiosciences, San Diego, CA) and 0.15 µM propidium iodide (Sigma-Aldrich). Cells were then diluted to 1–2 × 10⁶ cells/ml and fixed with 1% paraformaldehyde. Ten thousand events were run on the flow cytometer (Cytomics Flow Cytometer 500; Beckman Coulter) with excitation 488 and emission 525 (FITC) and 620 (propidium iodide).

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Data and Statistical Analyses

Data are expressed as means ± SE. For vasoreactivity measurements, Student’s t-tests (paired or unpaired) were used to compare dilator responses. To test for overall curve effect, we compared effective concentration 50% (EC₅₀), the dose of acetylcholine or bradykinin that produced 50% maximum dilation. EC₅₀ was derived using nonlinear regression using variable slope (four parameters) and least-squares (ordinary) fit (GraphPad Prism 5.0; GraphPad Software, San Diego, CA). For some LC-treated arterioles whose dilations to 10⁻⁴ M acetylcholine or 10⁻⁶ M bradykinin were <50% and could not be fitted in the model, EC₅₀ was assigned as 10⁻⁴ or 10⁻⁶ M, respectively (maximum dose used). Fluorescent values are expressed relative to vehicle control and compared and analyzed using paired Student’s t-test or one-way analysis of variance with Bonferroni posttest. NO levels were compared using paired Student’s t-test.
RESULTS

Adipose Arterioles

Baseline adipose diameter was 178.4 ± 9.3 μM (minimum 100.6, maximum 345.9, median 166.8 μM). There was reduced dilation to increasing doses of acetylcholine in adipose arterioles following 1 h exposure to 20 μg/ml LC (Fig. 1A and Table 1). There was a smaller reduction in dilator response to papaverine (81.4 ± 4.6 vs. 94.8 ± 1.3%, P = 0.006). There was no difference in the dilator response to acetylcholine [EC50: −4.5 ± 0.4 vs. −4.5 ± 1.3, P = not significant (NS)] or papaverine (85 ± 1 vs. 75 ± 18%, P = NS) between kappa vs. lambda LC-treated adipose arterioles. Cotreatment with PEG-SOD restored dilation to acetylcholine and papaverine (Fig. 1A and Table 1). LC caused increased superoxide and peroxynitrite production in adipose arterioles with attenuated NO production (Fig. 1, B–D). Cotreatment with PEG-SOD reversed the LC-induced increase in superoxide and peroxynitrite but did not attenuate NO reduction.

Table 1. Adipose arteriole vasoreactivity

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Dilation with 10−4 M Acetylcholine, %</th>
<th>EC50 Acetylcholine, log M</th>
<th>Dilation with 10−6 M Papaverine, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>16</td>
<td>85.8 ± 2.2</td>
<td>−6.9 ± 0.3</td>
<td>94.8 ± 1.3</td>
</tr>
<tr>
<td>LC</td>
<td>16</td>
<td>41.6 ± 7***</td>
<td>−4.2 ± 0.4***</td>
<td>81.4 ± 4.6**</td>
</tr>
<tr>
<td>LC + PEG-SOD</td>
<td>3</td>
<td>93.9 ± 8.5***</td>
<td>−6.4 ± 0.2++,++</td>
<td>99.6 ± 0.4</td>
</tr>
<tr>
<td>LC + tetrahydrobiopterin</td>
<td>4</td>
<td>82.1 ± 6.6+</td>
<td>−7.3 ± 0.5+</td>
<td>84.9 ± 5.5</td>
</tr>
<tr>
<td>LC + tetrahydrobiopterin + L-NAME</td>
<td>6</td>
<td>32 ± 7.5**</td>
<td>−3.2 ± 0.8###</td>
<td>51.7 ± 6.2***##</td>
</tr>
<tr>
<td>LC + mitoquinone</td>
<td>4</td>
<td>61 ± 9.3</td>
<td>−6.6 ± 2</td>
<td>75.3 ± 14.4</td>
</tr>
<tr>
<td>LC + gp91ds-tat</td>
<td>4</td>
<td>75 ± 10.5</td>
<td>−6.2 ± 0.8+</td>
<td>84.1 ± 8.4</td>
</tr>
<tr>
<td>Multiple myeloma LC</td>
<td>3</td>
<td>91.3 ± 7.5+</td>
<td>−6.8 ± 0.6+</td>
<td>91.6 ± 7.5</td>
</tr>
<tr>
<td>Denatured LC</td>
<td>3</td>
<td>71.4 ± 15</td>
<td>−5.8 ± 1.1</td>
<td>95.7 ± 1.1</td>
</tr>
</tbody>
</table>

Values are means ± SE. LC, light chain proteins; PEG-SOD, polyethylene glycol-superoxide dismutase; L-NAME, Nω-nitro-L-arginine methyl ester. *P < 0.05, **P < 0.01, and ***P < 0.001 vs. control. +P < 0.05 and ++P < 0.01 vs. LC. #P < 0.01 vs. LC + tetrahydrobiopterin. Denatured LC were subjected to 140°C heat for 1 h.
Coadministration of the NO synthase cofactor tetrahydrobiopterin with LC restored dilation to acetylcholine (Table 1), which could then be abolished with the NOS inhibitor L-NAME.

Coadministration of the mitochondrial antioxidant mitoquinone partially restored the dilator response to acetylcholine in the presence of LC (Table 1). Coadministration of the NADPH oxidase inhibitor gp91ds-tat partially restored the dilator response to acetylcholine in the presence of LC (Table 1).

To assess whether the dilator effects are related to protein tertiary or quaternary structure, the dilator response in adipose arterioles (n = 3) to acetylcholine was measured at baseline and following administration of LC that have been exposed to 140°C for 1 h, a thermal condition that has been found to provide irreversible denaturation and prevention of amyloid protein fibril formation (4). There was no difference in EC50 or dilution in response to acetylcholine or to papaverine vs. vehicle control (Table 1). There was no difference in the dilator response to acetylcholine or papaverine between LC derived from nonamyloid multiple myeloma patients and vehicle control, with a greater dilator response to acetylcholine compared with LC from AL subjects (Table 1). Unlike LC from AL subjects, nonamyloid LC did not increase superoxide production in adipose arterioles (0.78 ± 0.2 fluorescence relative to control, P = NS, n = 4).

**Coronary Arterioles**

Baseline coronary arteriole internal diameter was 160.9 ± 16 μM (minimum 84.2, maximum 325.9, median 140 μM). Coronary arterioles treated with LC showed reduced dilation to bradykinin (dilation at 10−6 M of 68.6 ± 6.2 vs. 90.9 ± 1.6% vehicle control, P < 0.001; log EC50 7.5 ± 0.4 vs. −8.4 ± 0.3, P = 0.03) (Fig. 2A). Dilator responses to papaverine were similar between LC and control arterioles. There was no difference in the dilator response to bradykinin (EC50: 7.5 ± 0.4 vs. −7.5 ± 1.4, P = NS) or papaverine (92 ± 1 vs. 80 ± 14%, P = NS) between kappa vs. lambda LC-treated coronary arterioles. Similar to adipose arterioles, LC increased superoxide and peroxynitrite production in coronary arterioles (Fig. 2, B and C).

**Human Coronary Artery Endothelial Cells**

One hour exposure to LC resulted in increased superoxide and peroxynitrite production in HCAECs (Fig. 3, A and B). HCAECs treated with LC plus mitoquinone, gp91ds-tat, or tetrahydrobiopterin showed no significant difference in either superoxide or peroxynitrite production compared with vehicle control while showing significantly lower levels compared with LC-treated cells. Analyses of Western blots showed no difference in total eNOS and the phospho-eNOS-to-total eNOS ratio between LC and vehicle-treated cells (Fig. 3, C and D). Twenty four hours of exposure of HCAECs to LC showed reduced NO head gas production compared with control, whereas tetrahydrobiopterin cotreatment blunted the NO reduction (in parts-billion−1·10−6 cells: vehicle control 22.3 ± 5.0, LC 9.6 ± 1.1, LC + tetrahydrobiopterin 12.6 ± 1.5, vehicle vs. LC P = 0.002, vehicle vs. LC + tetrahydrobiopterin P = NS, LC vs. LC + tetrahydrobiopterin P = NS).

Twenty four hours of LC treatment resulted in increased HCAEC staining with annexin-V (apoptosis marker) and propidium iodide (necrosis marker) (Fig. 4, A–E). Cotreatment with PEG-SOD prevented the increase in annexin-V and propidium iodide.

**DISCUSSION**

We present two novel findings. First, brief, acute exposure to AL amyloidosis LC induces microvascular dysfunction (endothelial and nonendothelial dysfunction in human adipose arterioles and endothelial dysfunction in coronary arterioles). Second, LC induce apoptotic and necrotic injury to HCAEC. The mechanism linking the microvascular dysfunction and apoptotic/necrotic injury induced by LC is likely related to oxidative stress (increased superoxide production), leading to reduced NO bioavailability and increased peroxynitrite production. This is the first study in a human tissue model demonstrating microvascular toxicity induced by LC.

AL is a multiorgan disease associated with poor prognosis, especially in the presence of cardiovascular involvement. Despite extensive use of chemotherapy (with or without autologous stem cell transplantation) to remove clonal plasma cells producing the LC, 12–18 mo mortality remains high (30–44%) (5, 23). Current treatment paradigms for AL amyloidosis do not address tissue toxicity induced by LC. This is likely because the underlying mechanisms of LC toxicity remain poorly understood. Animal studies implicate direct toxicity of LC on cardiomyocyte function and viability (8, 19, 32). The role of microvascular dysfunction in AL amyloidosis pathophysiology is not well known; in recent years, in another more
prevalent amyloid model, there is increasing recognition that cerebral microvascular dysfunction may be as important in causing Alzheimer’s morbidity as the toxic effects of amyloidogenic Aβ-proteins on neuronal cells (1). There is only scarce clinical evidence that microvascular dysfunction may be important in AL pathology. Endothelial dysfunction in medium- and small-sized vessels has been reported in patients with AL amyloidosis (6, 24). AL patients often present with angina and have impaired myocardial flow reserve because of small vessel involvement (21, 26). There is histological evidence of myocardial ischemia in 74% of patients (27). In the gut, all colon biopsy samples in one study revealed LC in the perivascular wall and stroma, associated with accumulation of lipid peroxidation products around the LC deposits, suggesting oxidative injury (2).

Our results confirm that acute exposure of both adipose and coronary arterioles to LC results in endothelial dysfunction, pointing to toxic effects of circulating proteins even without amyloid fibril deposition or other chronic components of the disease. This is also consistent with clinical observations that clearance of circulating LC following chemotherapy results in clinical improvement (11, 17) and prolonged survival (10) despite persistence or even an increase in the amyloid deposit seen on follow up biopsy (17). Our study did not address the role of deposited amyloid LC on endothelial function, especially in relation to circulating LC, an important field of study for the future. Thermal denaturation through exposure to 140°C heat reversed the ability of LC to induce adipose endothelial and nonendothelial arteriole dysfunction, suggesting that tertiary and quaternary structure may be important in its pathophysiology. Consistent with this is our finding of a normal dilator response to acetylcholine and papaverine and no increase in superoxide production when adipose arterioles were exposed to nonamyloid multiple myeloma LC, a differential response compared with amyloidogenic LC that was also observed in isolated rat cardiomyocytes (8).

We observed a similar response between adipose and coronary arterioles to LC with regard to endothelial dysfunction, pointing to a possible mechanism of multiorgan injury in different tissue beds. However, we only observed endothelium-independent (vascular smooth muscle) dysfunction in adipose arterioles. This may indicate tissue variability in the subendothelial effects of LC or reduced penetration because of the limited duration of exposure, although this should be confirmed by using dose-response studies of papaverine or other agents such as the NO donor sodium nitroprusside to fully evaluate nonendothelial function. Our findings of mild impairment of a dilator response to papaverine in adipose arterioles is
consistent with our previous observation showing an impaired response to the NO donor sodium nitroprusside (22).

Our results suggest possible mechanisms underlying LC microvascular toxicity. Increased superoxide in adipose and coronary arterioles and HCAECs and restoration of endothelial function and reduction of apoptotic and necrotic injury with antioxidant PEG-SOD strongly suggest induction of oxidative stress by LC. Endothelial dysfunction is likely caused by reduced NO bioavailability as shown by reduced NO in adipose arterioles and HCAECs. Reduced NO production was not associated with reduced eNOS or phospho-eNOS production in HCAECs. This is likely the result of reduced NO through reaction with superoxide generating peroxynitrite (supported by increased peroxynitrite in adipose and coronary arterioles and HCAECs). In addition, this may also be because of eNOS uncoupling leading to reduced NO production and increased superoxide production. This is supported by our findings showing restoration of adipose endothelial function when LC is coadministered with tetrahydrobiopterin, an essential cofactor for eNOS function and maintenance of eNOS coupling (20, 25); furthermore, in HCAECs, LC cotreated with tetrahydrobiopterin showed no significant difference in superoxide, peroxynitrite, and NO production with control. We demonstrated that the protective effect of tetrahydrobiopterin is through its effects on eNOS and not by a direct dilator effect because L-NAME abolished the beneficial effect on restoring endothelial function. Although eNOS uncoupling can be a source of LC-induced superoxide production, other potential sources may include mitochondria, NADPH oxidase, and xanthine oxidase. Partial, not full, restoration of endothelial function with mitoquinone cotreatment, a mitochondrially targeted antioxidant, suggests that mitochondria may be one but not the only source of superoxide. gp91ds-tat partially restored the dilator response to acetylcholine, suggesting that NADPH

![Fig. 4. HCAEC flow cytometry. A–C demonstrate flow cytometry of HCAECs treated with vehicle control, LC, and LC with PEG-SOD. D shows increased cells with annexin-V staining, and E shows increased cells with propidium iodide staining following 24 h LC exposure vs. vehicle control. Cotreatment with PEG-SOD reversed the increase in annexin-V and propidium iodide.](image)

![Fig. 5. Proposed schema of light chain protein-induced microvascular dysfunction and apoptotic injury.](image)
oxidase may be another source of superoxide production. It is possible that the doses of mitoquine and gp91ds-tat could be altered to achieve full preservation of endothelial function, and dose optimization could be pursued in future studies. The reason behind the abolition of LC-induced superoxide production with either mitoquine or gp91ds-tat alone is not known. It is possible that LC cause sequential ROS activation rather than parallel ROS production. The interplay and sequential signaling of ROS/reactive nitrogen species induced by LC need to be elucidated further in future studies. Finally, the link between endothelial dysfunction and cellular injury induced by LC is likely through reduced NO and increased peroxynitrite generation. Peroxynitrite is one of the most potent mediators of DNA and protein damage (28) and has been shown to induce apoptosis in multiple cell types, including vascular smooth muscle cells (18, 34). Peroxynitrite is a powerful oxidant of target molecules such as proteins (transition metal centers, amino acids, specifically, tyrosine nitration), lipids, and nucleic acids (28). The reduction in apoptotic and necrotic injury in HCAECs exposed to LC with PEG-SOD cotreatment is likely the result of reduced superoxide generation, reduced reaction with NO that simultaneously leads to decreased peroxynitrite, and enhanced NO bioavailability.

One interesting observation is the reduction of the dilator response to papaverine when adipose arterioles were exposed to the combination of LC, tetrahydrobiopterin, and l-NAME. Although the dilator effect of papaverine is thought to be NO-independent, a study by Jahr and colleagues (14) showed that l-NAME attenuated the dilator effects of papaverine in rat hindlimb arteries, suggesting possible interaction between NO suppression and papaverine action. The role of LC in modifying the tissue response to papaverine needs to be elucidated further in future studies.

Our findings of LC-induced oxidative and nitrosative stress in human arterioles and HCAECs are consistent with our previous finding of systemic oxidative stress in AL subjects with markedly increased levels of serum protein carbonyl (oxidized proteins) compared with control subjects (22). Pathology and histological examination of multiple organs in AL subjects also demonstrate evidence of oxidative stress and ischemic injury (2, 3, 12, 27, 35).

Based on our findings, we propose a novel model of microvascular injury induced by LC (Fig. 5). This novel pathway of injury may be the basis for future investigation of potential therapies directly addressing acute tissue toxicity of LC.

Limitations

The data are preliminary, and the results need to be validated in a larger sample of LC from patients with various organ involvement. The study is limited in studying only the acute effects of LC exposure on adipose and coronary arteriole function. We need further experimental data to determine whether the microvascular dysfunction is persistent and worsens with chronic exposure, since it is possible that compensatory mechanisms may be upregulated in response to LC exposure. The findings only address effects of LC whose contribution to pathology needs to be tested relative to deposited amyloid fibril proteins. Although our findings of LC effects on NO, superoxide, peroxynitrite, and-phospho-eNOS in addition to the response to cotreatment with tetrahydrobiopterin support eNOS uncoupling, we lack a measure of eNOS enzymatic activity that could further differentiate how eNOS is affected by LC.

Further mechanistic studies need to be performed as to specific signaling mechanisms by which LC induce oxidative stress, the sources of ROS, and the pathways inducing apoptotic and necrotic injury.

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Current address for S. Phillips: Department of Physical Therapy, University of Illinois at Chicago, Chicago, IL.

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DISCLOSURES

No conflict exists.

REFERENCES


