Primary (AL) amyloidosis is a plasma cell dyscrasia resulting in the clonal production of immunoglobulin light chain proteins (LC) and subsequent multi-organ dysfunction. Congestive heart failure remains the greatest cause of death in AL amyloidosis, due to the development of a rapidly progressive amyloid cardiomyopathy. Patients with amyloid cardiomyopathy are largely unresponsive to current heart failure therapies\(^2,3\) and have a median survival of less than 6 months and a 5-year survival of less than 10\%. The mechanisms underlying this disorder, however, have yet to be determined. Prior theories have suggested that interstitial fibril deposition of AL proteins are the main cause of contractile dysfunction and cardiomyopathy.\(^2\) This, however, is inconsistent with clinical observations, which have detailed a lack of correlation between myocardial fibril deposition and cardiac dysfunction in AL patients.\(^3\) In addition, recent work in noncardiac tissues have suggested that other amyloidogenic proteins, including Aβ and transthyretin, may directly impair cellular function, independent of fibril deposition, through redox sensitive mechanisms.\(^5,7\)

In this report, we demonstrate that physiological levels of human amyloid LC proteins, isolated from patients with amyloid cardiomyopathy (cardiac-LC), specifically alter cellular redox state in isolated cardiomyocytes, marked by an increase in intracellular reactive oxygen species and upregulation of the redox-sensitive protein, heme oxygenase-1. In contrast, vehicle or control LC proteins isolated from patients without cardiac involvement did not alter cardiomyocyte redox status. Oxidant stress imposed by cardiac-LC proteins further resulted in direct impairment of cardiomyocyte contractility and relaxation, associated with alterations in intracellular calcium handling. Cardiomyocyte dysfunction induced by cardiac-LC proteins was independent of neurohormonal stimulants, vascular factors, or extracellular fibril deposition, and was prevented through treatment with a superoxide dismutase/catalase mimetic. This study suggests that cardiac dysfunction in amyloid cardiomyopathy is directly mediated by LC protein-induced cardiomyocyte oxidant stress and alterations in cellular redox status, independent of fibril deposition. Antioxidant therapies or treatment strategies aimed at eliminating circulating LC proteins may therefore be beneficial in the treatment of this fatal disease.

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Results
Cardiac-LC Increase Intracellular ROS- and Redox-Sensitive Protein Expression
To determine whether physiological levels of LC directly alter redox status in cardiomyocytes, intracellular ROS levels were determined after exposure to amyloidogenic proteins. As shown in Figure 1A, human Con-LC proteins did not influence ROS levels relative to vehicle exposure. In contrast, Cardiac-LC proteins directly induced cellular oxidant stress, as demonstrated by enhanced DCF fluorescence. Elevated ROS levels in Cardiac-LC cells were prevented through antioxidant treatment with MS-TMPyP.

Redox-sensitive protein expression was also determined after incubation with LC, using the cellular stress marker, HO-1.\(^10\) Cardiac-LC resulted in an upregulation of HO-1 expression relative to vehicle and Con-LC in a dose-dependent manner (Figure 1B).
Interestingly, even low levels of Cardiac-LC (10 mg/L) increased HO-1 expression, and this effect was augmented greater than 2-fold with physiological concentrations of LC protein. In addition, HO-1 induction increased in a time-dependent manner with Cardiac-LC (online Figure available in the online data supplement), with augmented expression as early as 6 hours after incubation. Induction of HO-1 was prevented through antioxidant treatment with the superoxide dismutase/catalase mimetic, MnTMPyP, confirming redox-sensitive expression (Figure 1B).

Importantly, LC proteins did not form either LC aggregates or amyloid fibrils, as determined by spectrophotometric analysis8 (Figure 1C) or Congo red staining (data not shown), suggesting that Cardiac-LC directly altered redox state in cardiomyocytes, independent of fibril formation.

Cardiac-LC Impairs Cardiomyocyte Contractility and Intracellular Calcium Handling

We subsequently determined the effects of LC and altered redox state on cardiomyocyte contractility and intracellular calcium transients under physiological conditions. Cardiac-LC directly reduced cardiomyocyte cell shortening (Figure 2A) and prolonged cellular relaxation (Figure 2B), whereas Con-LC did not alter cellular function. As shown in Figure 2C, decreased cell shortening with Cardiac-LC was accompanied by a concomitant decrease in both peak intracellular calcium levels and calcium transient amplitude, thereby suggesting that Cardiac-LC mediated contractile dysfunction was secondary to impaired calcium handling. Interestingly, however, altered cellular relaxation with Cardiac-LC was not associated with prolonged intracellular calcium reuptake (Figure 2D).
2D), consistent with a calcium-independent mechanism for impaired relaxation. The effects of Cardiac-LC proteins on cardiomyocyte function and intracellular calcium were reversed to baseline levels with antioxidant treatment, suggesting that Cardiac-LC induced cellular dysfunction was secondary to increased oxidant stress. Representative tracings of cell shortening and intracellular calcium transients are shown in Figure 2E.

Discussion
Amyloid cardiomyopathy represents a rapidly progressive and fatal form of cardiomyopathy. The mechanisms underlying this disease remain unknown. In this report, we demonstrate that human amyloidogenic cardiac-LC proteins specifically alter cellular redox state in isolated cardiomyocytes, resulting in direct impairment of cardiomyocyte contractile function and calcium handling.

This is the first report to document a direct effect of cardiac-LC on cardiomyocyte redox status and contractile function. Decreased cellular shortening in cardiomyocytes with cardiac-LC treatment was accompanied by a decrease in calcium release during contraction. Impaired cellular relaxation, however, was not associated with slowed calcium reuptake. Therefore, whereas dysfunction of cardiomyocyte contraction and relaxation were both redox-sensitive and reversed with antioxidant treatment, this dysfunction was likely secondary to intracellular calcium-dependent as well as calcium-independent mechanisms. Impaired cellular function may represent oxidant stress-mediated modification of redox-sensitive calcium handling proteins and/or myofilament proteins. Our observation of impaired contraction and relaxation in cardiomyocytes is in contrast to prior work detailing an effect of cardiac LC only on diastolic function in isolated hearts. This discrepancy, however, is likely secondary to differences in methodology (loaded isovolumically-contracting hearts versus unloaded cardiomyocytes) and cardiac-LC exposure time in isolated hearts versus cardiomyocytes (30 minutes versus 24 hours, respectively).

Importantly, the effects of cardiac-LC occurred in the absence of neurohormonal/vascular factors or extracellular fibril deposition, previously believed to be central to the pathogenesis of amyloid cardiomyopathy. As such, these results may explain clinical observations detailing a discrepancy between the degree of myocardial cardiomyopathy. As such, these results may explain clinical observations detailing a discrepancy between the degree of myocardial cardiomyopathy and cardiac dysfunction. Interestingly, only LC proteins associated with cardiomyopathy, rather than noncardiac-associated LC proteins, resulted in increased ROS and cardiomyocyte dysfunction in isolated cells, suggesting that LC primary sequence and/or posttranslational modifications, rather than osmolar stress or nonspecific protein-receptor interaction, dictate end-organ targeting and dysfunction. Moreover, significant cellular oxidant stress and cardiomyocyte dysfunction were observed with physiological concentrations of LC proteins, and with proteins isolated from multiple patients, thereby eliminating artifact associated with supranormal protein concentrations or single protein variants. These results are consistent with prior findings documenting direct impairment of cellular function, independent of fibril deposition, in noncardiac tissue by other amyloidogenic proteins.

The heightened sensitivity of cardiomyocytes to oxidant injury has been well documented, and the role of ROS in the pathogenesis of myocardial failure continues to expand. Furthermore, emerging evidence suggests that increased oxidant stress and oxidative cellular injury contribute significantly to the pathophysiology of other amyloidogenic disease, notably Alzheimer’s disease. The mechanisms by which amyloid cardiac-LC proteins increase cellular ROS alter redox state may include activation of cellular oxidase complexes, mitochondrial dysfunction, or metal ion reduction.

This study suggests for the first time that cardiac dysfunction in amyloid cardiomyopathy is directly mediated by LC protein-induced cardiomyocyte oxidant stress and alterations in cellular redox status, independent of fibril deposition. Antioxidant therapies or treatment strategies aimed at eliminating circulating LC proteins may therefore be beneficial in the treatment of this fatal disease.

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References

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Supplemental Materials and Methods

Light Chain Isolation and Purification

Human LC proteins were isolated from patients referred to the Amyloid Treatment and Research Program at Boston University School of Medicine with non-amyloidogenic myeloma (n=1), non-cardiac involved AL amyloidosis (n=1), or cardiac-involved AL amyloidosis (n=2)(Cardiac-LC), as previously described. Briefly, urine samples, collected from patients, were dialyzed against deionized water, lyophilized, and reconstituted in 0.02 mol/L sodium-phosphate buffer (pH 7.1) with Affigel blue (Bio-Rad) to remove albumin. The entire filtrate was again dialyzed and lyophilized. The affinity-purified sample was fractionated on Sephacryl S-200 columns (Amersham-Pharmacia) in 0.02 mol/L Tris (pH 7.5). The purity of fractionated samples was assessed by SDS-PAGE and Western blotting using the Pharmacia Phast System. Results obtained with non-amyloidogenic and non-cardiac involved amyloidosis proteins were similar, and as such, results were grouped together and presented as a single control protein group (Con-LC). All samples were collected with Institutional Review Board approval and informed consent.

Cardiomyocyte Culture and Treatment

This study was performed in accordance with the guidelines of the Animal Care and Use Committee of Boston University School of Medicine and the National Institutes of Health Guide for Care and Use of Laboratory Animals. Adult rat ventricular cardiomyocytes were isolated using collagenase perfusion, as previously described.
Cells were plated on laminin (1 µg/cm²)-coated dishes at a density of 100 cells/mm² for Western blotting and fluorescence studies, and on glass coverslips for cell contractility and calcium transient measurements. Cardiomyocytes were treated with ultrapurified water (vehicle), Con-LC, or Cardiac-LC at physiologic concentrations (20 mg/L) for 24 hours, unless otherwise specified. For antioxidant experiments, cardiomyocytes were cotreated with the superoxide dismutase/catalase mimetic Mn(III)terakis(1-methyl-4-pyridyl) porphyrin pentachloride (MnTMPyP) (Calbiochem) at 50 µmol/L.

**Measurement of Intracellular ROS**

Intracellular ROS levels were determined using the cell-permeable, redox-sensitive fluorophore, dichlorofluorescein-diacetate (DCF) (Molecular Probes), as previously described.² Oxidation of DCF by intracellular ROS results in the formation of a fluorescent compound. Cardiomyocytes were incubated with 20 µmol/L DCF for 30 minutes and DCF fluorescence visualized (excitation 308nm, emission 510nm) and quantified using epi-fluorescent microscopy and video imaging (BIOQUANT, version 2.5).

**Western Blotting**

Protein expression of heme oxygenase-1 (HO-1) was determined through Western blot.² Briefly, cardiomyocytes were lysed using a 0.1% Triton Buffer (Cell Signaling) and sonicated. Total protein was determined according to the methods of Lowry ³. Samples were mixed with β-mercaptoethanol, thermally denatured at 90°C for 3 minutes, and separated by SDS-PAGE (50µg/well). Proteins were transferred to PVDF membrane
(Bio-Rad) and blocked with 5% non-fat dry milk in 0.1% Tween-TBS for 1 hour. Immunodetection of HO-1 was determined using polyclonal rabbit anti-rat HO-1 antibody (Stressgen) at a concentration of 1:3000 followed by 1 hour incubation with HRP labeled anti-rabbit IgG secondary antibody (Pierce) at a concentration of 1:5000. Membranes were exposed using enhanced chemiluminescence and HO-1 levels determined by densitometry.

**Light Chain Aggregation and Amyloid Fibril Formation**

Light chain aggregation was determined through absorbance spectrometry. Following 24 hours cardiomyocyte incubation with light chains, phenol-free culture media was aspirated. The sample aspirate was vortexed to resuspend any precipitate and light chain aggregation determined through assessment of turbidity at 400 nm using a Beckman UV spectrometer. Amyloid fibril formation was determined through Congo-red staining and visualization of apple-green birefringence under polarized light microscopy, as previously described.

**Cell Contractility and Intracellular Calcium Transients**

Cell shortening and intracellular calcium transients ([Ca^{2+}]_{i}, transients) were recorded in cardiomyocytes, as previously described. Cultured cardiomyocytes were incubated with membrane permeant fura-2 (1 µmol/L) (Molecular Probes) and probenecid (500 µmol/L), to prevent leakage of fura-2 from cells. Cardiomyocytes were maintained at 37°C, perfused with 1.2 mmol/L Ca^{2+} Tyrode solution, and electrically paced at 5 Hz via platinum wires. Cell shortening/relengthening, and [Ca^{2+}]_{i}, transients
were measured using video edge detection and fluorescence measurements of the Fura-2 ratio, respectively (SoftEdge Acquisition System and IonWizard, IonOptix Inc). Percent cell shortening (% CS) was calculated as diastolic cell length minus systolic cell length normalized to the diastolic cell length. The time constant (τ), a cell length–independent measure of cardiomyocyte relaxation, was calculated as previously described \(^2\) using commercially available acquisition software (IonWizard, IonOptix Inc). \([\text{Ca}^{2+}]_i\) was calculated from fura-2 fluorescence as previously described,\(^2\) and \([\text{Ca}^{2+}]_i\) transients were analyzed in a similar fashion to cell shortening experiments.

**Statistical Analysis**

All data are presented as mean±SE. Group comparisons were made using ANOVA with a post-hoc test of least significant differences. A p-value <0.05 was considered statistically significant.
Supplemental References


Supplemental Figure Legends:

**Supplemental Figure:** Time-dependent upregulation of heme oxygenase-1 (HO-1) with exposure to human cardiac-LC proteins. Time-dependent representative and quantitative Western blot of the redox-sensitive protein, HO-1 following exposure to vehicle, Con-LC, Cardiac-LC, or Cardiac-LC + MnTMPyP. *: p<0.05 vs vehicle, †: p<0.05 vs Con-LC; #: p<0.05 vs Cardiac-LC.