PB069

Aggregated Light Chain Increases Brain Natriuretic Peptide Production and Induces Oxidative Stress Response in Cardiomyocytes

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INTRODUCTION

- Amyloid light chain (AL) amyloidosis is a rare, progressive, and typically fatal protein misfolding disease caused by deposition of aggregated immunoglobulin light chain (LC) protein, which in turn forms soluble toxic aggregates and deposited fibrils (amyloid)[1-3]
  - LCs prone to misfolding are produced in excess by clonal plasma cells
  - Amyloid leads to progressive failure of vital organs, including the heart, kidneys, and nervous system, causing significant morbidity and mortality[1-3]
  - Early intervention is critical for patients with AL amyloidosis, particularly those with cardiac involvement (as many as 74% of patients)[4,5]
- There are no approved treatments for AL amyloidosis, though patients may be treated with off-label therapies directed at the underlying plasma cell dyscrasia[3,4,6]
  - Serum levels of the N-terminal fragment of the probrain natriuretic peptide (NT-proBNP) are used clinically to diagnose cardiac involvement and to stage patients, representing a key prognostic factor of survival[7]
  - Reductions in NT-proBNP levels after treatment indicate a cardiac organ response to treatment measured using consensus criteria; studies of patients with AL amyloidosis show cardiac response to be predictive of increased survival[7]
- It has been proposed that elevations in NT-proBNP levels in patients with AL amyloidosis and cardiac involvement result not only from mechanical strain, as in other forms of cardiomyopathy, but also from a direct cytotoxic effect of LCs on the p38 mitogen-activated protein kinase (MAPK) signaling pathway, increasing brain natriuretic peptide (BNP) synthesis by cardiomyocytes[8]
  - This suggests that changes in NT-proBNP levels in patients with AL amyloidosis have a clear link to disease mechanisms and so are a much stronger predictor of outcomes in patients with AL amyloidosis and cardiac involvement than in patients with other forms of heart failure
  - Understanding the mechanisms by which NT-proBNP is regulated is important clinically because this understanding is the basis by which prognosis and therapeutic response are determined

METHODS

Cell Culture

- Neonatal Wistar rat hearts were freshly dissociated and cultured using the primary cardiomyocyte isolation kit according to the manufacturer's instructions (Thermo Scientific Pierce). After 5 days, cultured cardiomyocytes were serum starved overnight and treated with Dulbecco-modified Eagle medium (DMEM) alone (vehicle control), 10 nM endothelin-1 (ET-1) (cardiac hypertrophy positive control), or a mixture of aggregated and monomeric λ LC aggregates (0.2 mg/mL) in serum-free DMEM. Conditioned media were then collected for NT-proBNP protein quantification and RNA purified for quantitative polymerase chain reaction (qPCR)
- Where indicated, the small molecule inhibitors tin protoporphyrin IX (heme oxygenase-1 [Hmox-1] inhibitor [Hmox-1i]) at 30 μM, tin mesoporphyrin IX (heme oxygenase-1 [Hmox-1] inhibitor [Hmox-1ib]) at 30 μM, SB203580 (p38 inhibitor [p38i]) at 5 μM, L-NMMA (endothelial nitric oxide synthase [eNOS] inhibitor) at 100 μM, and NS-2028 (soluble guanylyl cyclase inhibitor) at 10 μM were used to pretreat cardiomyocytes before LC aggregates were added

Recombinant Soluble and Insoluble LC Aggregates

- An AL amyloidosis–associated, full-length LC sequence (FL-λ6a) derived from a known patient sequence containing both constant and variable regions[9] was recombinantly expressed in Chinese hamster ovary cells and purified by affinity chromatography
- Aggregations were carried out by shaking unaggregated protein (0.75 mg/mL) at 500 rpm for 3-5 days at 48°C. Unaggregated and aggregated LCs were characterized by size-exclusion chromatography, thioflavin T fluorescence, and electron microscopy
- Aggregated LCs were separated by centrifugation into insoluble (pellet) and soluble (supernatant) forms

Quantitative Polymerase Chain Reaction

- Relative gene expression was analyzed using TaqMan Gene Expression Master Mix and Assays (Applied Biosystems; MGB probes, FAM dye labeled) according to the manufacturer's instructions

MSD Electrochemiluminescence Assay

- Rat NT-proBNP was quantified using a rat-specific NT-proBNP kit according to the manufacturer's specifications (Meso Scale Discovery [MSD])
- Cell surface–bound FL-λ6a LC was detected using a SULFO-TAG (MSD)–labeled anti–λ LC rabbit polyclonal antibody in a 96-well High Bind MSD plate for 2 hours at 37°C and 5% CO_2 (Dako Agilent Technologies)

Measurement of ROS

- Effects of FL-λ6a and ET-1 on reactive oxygen species (ROS) were measured using the ROS-Glo H_2O_2 Assay (Promega Biotechnology) according to the manufacturer's specifications

Flow Cytometry

- 1 mg/mL aggregated LC in phosphate-buffered saline was biotinylated according to the manufacturer's instructions using a fivefold molar excess of NHS-PEG4-Biotin compared with agg-LC
- 600,000 primary rat cardiomyocytes were added to LC, shaken for 10 seconds, and inclubated for 30 minutes at 4ºC; after two washes, 0.5 ng/μL Streptavidin-conjugated allophycocyanin (APC) was added to the cells and shaken and incubated again; APC fluorescence was detected by flow cytometry using standard procedures (FACSAria II; BD Biosciences)

Statistical Analysis

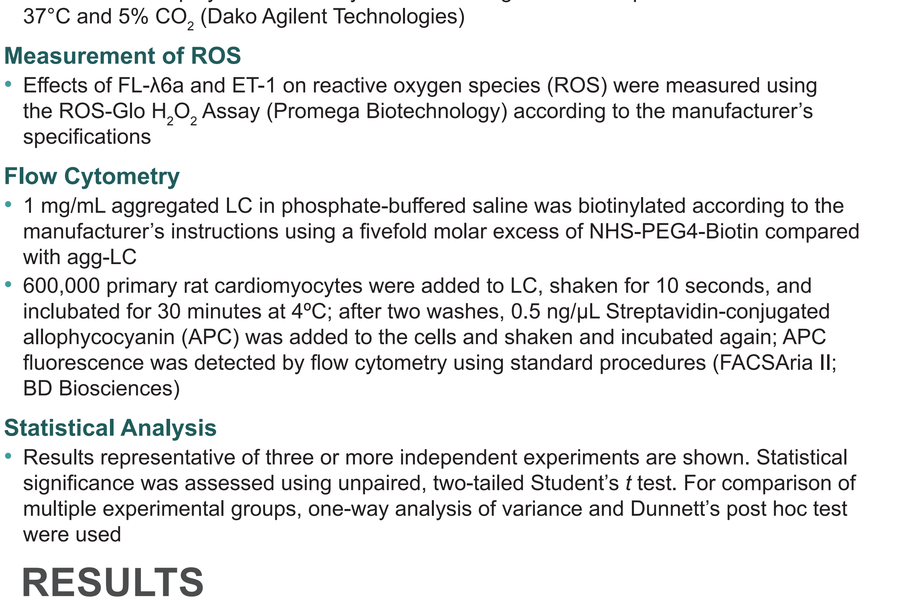
- Results representative of three or more independent experiments are shown. Statistical significance was assessed using unpaired, two-tailed Student's *t* test. For comparison of multiple experimental groups, one-way analysis of variance and Dunnett's post hoc test were used

RESULTS

LC Aggregates Induced Cellular Oxidative Stress in Cardiomyocytes

- Treatment with patient-derived LC aggregates increased transcript levels of the oxidative stress response markers Hmox-1 and Egr-1 after 4 hours of treatment (**Figures 1A-B**)
- Intracellular H_2O_2 levels were increased in response to LC aggregates (**Figure 1C**)
- These cellular effects are likely associated with LC aggregate binding to cardiomyocyte membranes as measured using flow cytometry (**Figure 1E**), which was concentration dependent, as indicated by an electrochemiluminescence assay (**Figure 1D**) and by flow cytometry (**Figure 1F**)
- In contrast, treatment with ET-1, a mediator of robust cardiac hypertrophic response, did not modulate oxidative stress response marker expressions or ROS (**Figures 1A-C**)
  - These results suggest that LC aggregates and cardiac hypertrophy activate distinct intracellular signaling pathways in cardiomyocytes

Figure 1. (A-E) Patient-derived LC aggregate sequences increased expression of the oxidative stress response marker Hmox-1 and cellular ROS.

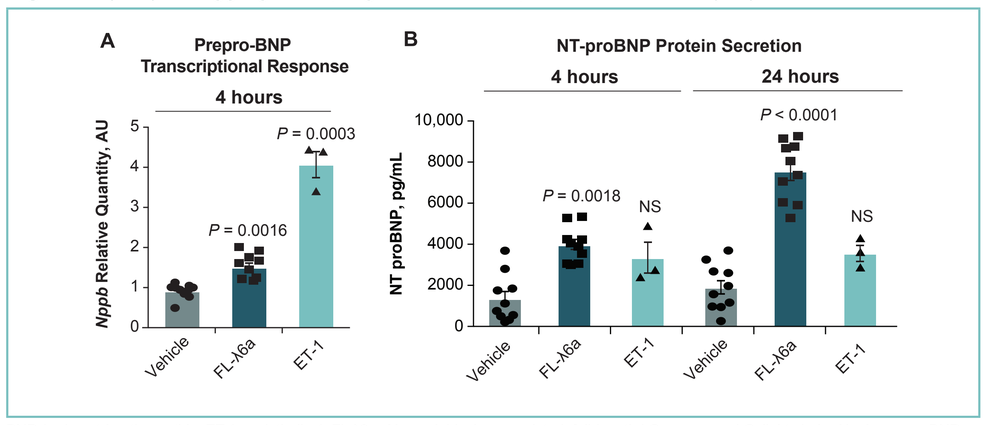


APC, allophycocyanin; ET-1, endothelin-1; Egr-1, early growth response-1; FL-λ6a, AL amyloidosis–associated, full-length LC sequence; FSC, forward scatter; H_2O_2, hydrogen peroxide (ROS); Hmox-1, heme oxygenase-1; LC, light chain; MSD, Meso Scale Discovery; NS, not significant; ROS, reactive oxygen species.
A-C: n = 10 (vehicle, FL-λ6a), n = 3 (ET-1).
All comparisons were performed with vehicle control. Mean fluorescence intensity was calculated from the average fluorescence of each cell. Error bars represent standard error of the mean. Data in panel E represent 50 μg/mL FL-λ6a.

LC Aggregates Induced BNP Production in Cardiomyocytes in a Hmox-1–Dependent Fashion

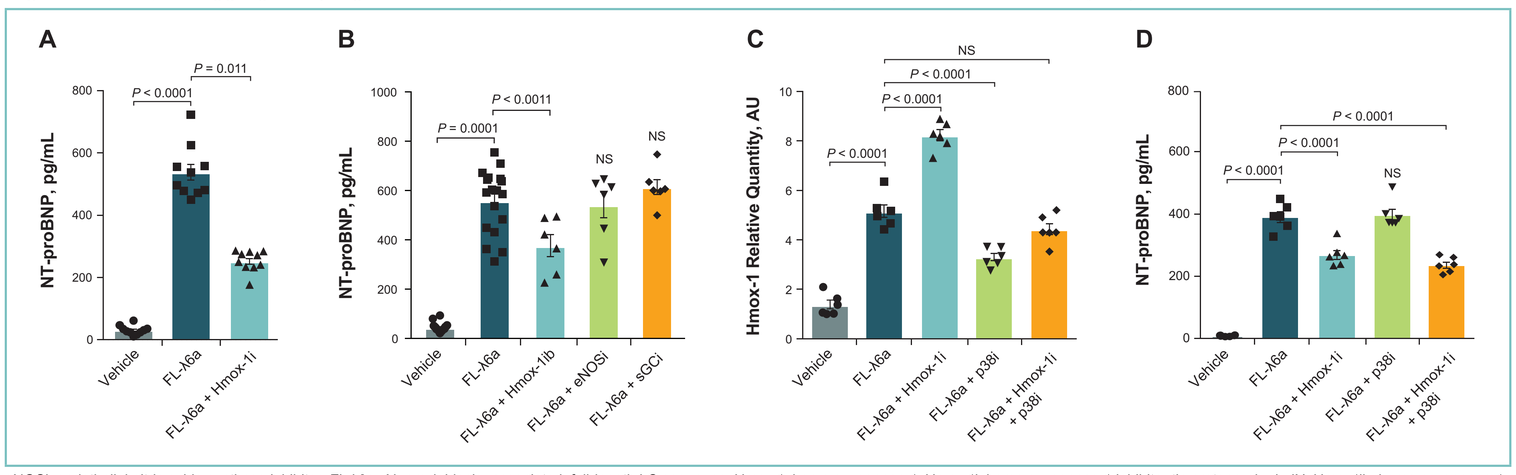
- Both cardiomyocyte BNP transcription and NT-proBNP protein secretion were robustly upregulated in response to LC aggregate treatment in as few as 4 hours (**Figures 2A-B**)
  - A more pronounced effect on NT-proBNP in the media suggested that LC aggregate treatment primarily activated NT-proBNP production through secretion (**Figures 2A-B**)
- Conversely, ET-1–induced BNP production exhibited the opposite phenomenon, whereby its effect on NT-proBNP protein secretion was considerably less than on BNP transcriptional upregulation (**Figures 2A-B**)
  - These results suggest that LC aggregates and cardiac hypertrophic activation drive increases in BNP levels along two distinct signaling pathways

Figure 2. (A-B) LC aggregates upregulated BNP expression in cardiomyocytes.



BNP, brain natriuretic peptide; ET-1, endothelin-1; FL-λ6a, AL amyloidosis–associated, full-length LC sequence; LC, light chain; *Nppb*, prepro-BNP; NS, not significant; NT-proBNP, N-terminal fragment of the probrain natriuretic peptide.
A-B: n = 10 (vehicle, FL-λ6a), n = 3 (ET-1).
Error bars represent standard error of the mean. All comparisons were performed with vehicle control.

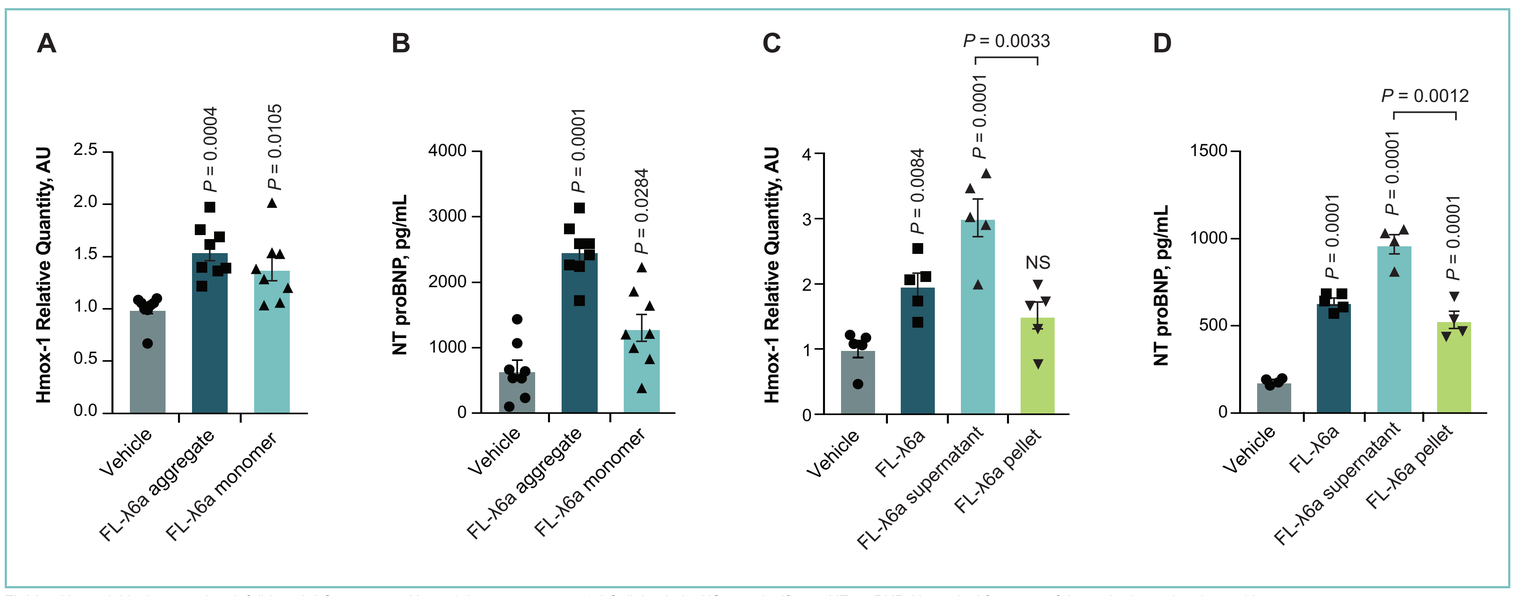
Figure 3. (A-D) LC aggregate upregulation of NT-proBNP secretion and Hmox-1 expression were differentially dependent on Hmox-1 and p38.



eNOSi, endothelial nitric oxide synthase inhibitor; FL-λ6a, AL amyloidosis–associated, full-length LC sequence; Hmox-1, heme oxygenase-1; Hmox-1i, heme oxygenase-1 inhibitor tin protoporphyrin IX; Hmox1ib, heme oxygenase-1 inhibitor tin mesoporphyrin IX; LC, light chain; NS, not significant; NT-proBNP, N-terminal fragment of the probrain natriuretic peptide; p38i, p38 inhibitor; sGCi, soluble guanylyl cyclase inhibitor.
A, C-D: n = 10; B, n = 6-12/group.
Error bars represent standard error of the mean.

- Hmox-1 linked LC aggregate–induced oxidative stress and downstream NT-proBNP production (**Figures 3A-D**)
  - A small molecule inhibitor of Hmox-1 catalytic activity attenuated LC aggregate–induced NT-proBNP production by ~60% (**Figure 3A**)
  - Attenuation in LC aggregate–induced NT-proBNP production was specific to Hmox-1 and not to eNOS or soluble guanylyl cyclase (**Figure 3B**)
  - Inhibition of p38 partially attenuated LC aggregate–induced Hmox-1 upregulation by ~40%. There was no additive effect of p38 inhibition and Hmox-1 inhibition (**Figure 3C**)
  - Conversely, LC aggregate–induced NT-proBNP production was unaffected by p38 MAPK inhibition, suggesting that partial blockade of LC aggregate–induced Hmox-1 upregulation is insufficient to inhibit downstream secretion of NT-proBNP. There was no additive effect of p38 inhibition and Hmox-1 inhibition (**Figure 3D**)

Figure 4. (A-D) Soluble LC aggregates drove the majority of BNP expression and NT-proBNP secretion in cardiomyocytes.



FL-λ6a, AL amyloidosis–associated, full-length LC sequence; Hmox-1, heme oxygenase-1; LC, light chain; NS, not significant; NT-proBNP, N-terminal fragment of the probrain natriuretic peptide.
A-B: n = 8/group; C, n = 5/group; D, n = 4/group.
Data represent 4 hours after treatment. All comparisons were performed with vehicle control unless specifically noted. Error bars represent standard error of the mean.

- LC aggregate promoted Hmox-1 transcriptional upregulation (**Figure 4A**) and increased levels of secreted NT-proBNP protein (**Figure 4B**) more robustly than LC monomer
- Soluble LC aggregates (supernatant) were more biologically active than insoluble LC aggregates (pellet) (**Figures 4C-D**)
  - These results suggest that soluble LC aggregates are the primary cardiotoxic species driving oxidative stress and downstream NT-proBNP production in AL amyloidosis

CONCLUSIONS

- Patient-derived LC aggregates can directly induce increases in both oxidative stress and BNP expression in primary rat cardiomyocytes. Furthermore, NT-proBNP secretion may be directly regulated by cardiotoxicity through Hmox-1 catalytic activity
- LC aggregates and ET-1 may modulate increases in NT-proBNP levels primarily through secretion or transcriptional upregulation, respectively
- Hmox-1 and p38 MAPK function as key signaling molecules in LC aggregate–induced cardiotoxicity and BNP production but may act through redundant signaling pathways
- Soluble LC aggregates were more potent in inducing insult than larger LC aggregates, though both monomeric and pelleted LC aggregates were still able to modestly upregulate Hmox-1 transcript and NT-proBNP secretion
- These results suggest that elevated levels of NT-proBNP in patients with AL amyloidosis may correlate with the degree of insult induced by LCs to cardiomyocytes and may contribute to disease activity
- Furthermore, these data support the notion that NT-proBNP may represent a direct measure of cardiotoxicity in AL amyloidosis, in contrast to other forms of heart failure (**Figure 5**), and support the use of NT-proBNP as a surrogate biomarker for therapeutic efficacy in clinical trials

Figure 5. LC aggregate–mediated induction of cellular oxidative stress directly upregulated BNP expression.

BNP, brain natriuretic peptide; Hmox-1, heme oxygenase-1; LC, light chain; MAPK, mitogen-activated protein kinase; NT-proBNP, N-terminal fragment of the probrain natriuretic peptide.

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Disclosures of Interest

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