Age-Independent Cardiac Protection by Pharmacological Activation of Beclin-1 During Endotoxemia and Its Association With Energy Metabolic Reprograming in Myocardium—A Targeted Metabolomics Study

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BACKGROUND: We showed that Beclin-1-dependent autophagy protects the heart in young and adult mice that underwent endotoxemia. Herein, we compared the potential therapeutic effects of Beclin-1 activating peptide, TB-peptide, on endotoxemia-induced cardiac outcomes in young adult and aged mice. We further evaluated lipopolysaccharide (lipopolysaccharide)-induced and TB-peptide treatment-mediated alterations in myocardial metabolism.

METHODS AND RESULTS: C57BL/6J mice that were 10 weeks and 24 months old were challenged by lipopolysaccharide using doses at which cardiac dysfunction occurred. Following the treatment of TB-peptide or control vehicle, heart contractility, circulating cytokines, and myocardial autophagy were evaluated. We detected that TB-peptide boosted autophagy, attenuated cytokines, and improved cardiac performance in both young and aged mice during endotoxemia. A targeted metabolomics assay was designed to detect a pool of 361 known metabolites, of which 156 were detected in at least 1 of the heart tissue samples. Lipopolysaccharide-induced impairments were found in glucose and amino acid metabolisms in mice of all ages, and TB-peptide ameliorated these alterations. However, lipid metabolites were upregulated in the young group but moderately downregulated in the aged by lipopolysaccharide, suggesting an age-dependent response. TB-peptide mitigated lipopolysaccharide-mediated trend of lipids in the young mice but had little effect on the aged. (Study registration: Project DOI: https://doi.org/10.21228/M8K11W).

CONCLUSIONS: Pharmacological activation of Beclin-1 by TB-peptide is cardiac protective in both young and aged population during endotoxemia, suggest a therapeutic potential for sepsis-induced cardiomyopathy. Metabolomics analysis suggests that an age-independent protection by TB-peptide is associated with reprogramming of energy production via glucose and amino acid metabolisms.

Key Words: autophagy; Beclin-1; cardiac function; cardiac metabolism; endotoxemia; sepsis

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Supplemental Material is available at https://www.ahajournals.org/doi/suppl/10.1161/JAHA.122.025310

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JAHA is available at: www.ahajournals.org/journal/jaha

J Am Heart Assoc. 2022;11:e025310. DOI: 10.1161/JAHA.122.025310
Sepsis is a life-threatening condition of organ dysfunction caused by a deregulated host response to infection. Despite improvements in antibiotic therapies and critical care techniques, sepsis remains a leading cause of death in critical care units, and its reported incidence is still increasing. Therefore, understanding the pathological mechanisms and exploring new therapeutic interventions for sepsis has become an urgent task.

Cardiomyopathy is an identified serious component of the multiorgan failure associated with sepsis. Energy expenditure is a main regulatory element of cardiac contractility, and metabolism changes dynamically with physiological and pathological conditions. The normal heart is equipped with a remarkable degree of metabolic flexibility, whereby ATP is rapidly supplied via multiple substrates, such as fatty acids, carbohydrates, ketones, and amino acids (AAs), to meet the energy demand. Failing in cardiac performance is often associated with metabolic inflexibility, under which condition the heart loses the capability of using certain commonly used substrates. In sepsis models, this problem of metabolic inflexibility is apparent in the heart, as well as in other organs and circulating immune cells. Current research in immunometabolism has revealed that disturbance in the energy metabolism of immune cells magnifies the adverse symptoms in sepsis. However, in additional to inciting overwhelming inflammation, how the disturbance of metabolic homeostasis in immune cells and in other cell types leads to multiorgan failure, such as cardiomyopathy, remains unclear.

In the heart, mitochondria occupy about 30% of the cardiomyocyte volume. Previous research in preclinical sepsis models elucidated that impairment in mitochondrial structure and function results in an overproduction of mitochondrial reactive oxygen species and a generation of mitochondria-derived danger-associated molecular patterns, inducing cardiac inflammation and functional deficiencies. As the main source of energy production in the heart, mitochondria supply 90% of the total ATP via metabolism of glucose, AAs, and fatty acids. Therefore, deficiencies in mitochondria are likely the main cause for metabolic inflexibility in septic hearts. A comprehensive understanding of alterations in mitochondria and related metabolic reprograming will help to identify novel therapeutic targets and to develop effective strategies for improving clinical outcomes.

We recently investigated the role of autophagy, a self-survival lysosome-dependent process, in the control of cardiac performance during endotoxemia. We discovered that promoting autophagy via specific activation of Beclin-1, a universally expressed autophagy initiation factor, improved cardiac contractility, protected mitochondria, and suppressed mitochondrial danger-associated molecular patterns in response to endotoxemia. Accordingly, we further examined the potential therapeutic value of TB-peptide, a cell-permeable peptide that specifically activates Beclin-1, in sepsis animal models using young adult mice. In the investigation summarized in this report, we further evaluated TB-peptide’s effects on cardiac function of aged animals during endotoxemia. In addition, we applied a targeted metabolomics approach to compare how lipopolysaccharide alters metabolic profiling in the heart of young adult and aged mice to examine whether TB-peptide reprograms cardiac metabolism in this animal model of endotoxemia.

**METHODS**

The data that support the findings of this study are available from the corresponding author upon reasonable request. The metabolomics analysis in this study is available at the National Institutes of Health Common Fund’s National Metabolomics Data Repository website (supported by National Institutes of Health grant U2C-DK119886), where it has been assigned Study ID ST002178. The data can be accessed directly via its Project DOI: https://doi.org/10.21228/M8K11W.

**Experimental Animals**

Wild-type C57BL/6 mice were obtained from Charles River laboratories (Wilmington, MA) and an in-campus...
mouse breeding core facility at the University of Texas Southwestern Medical Center. All animals were conditioned in house for 5 to 6 days after arrival with commercial diet and tap water available ad libitum. Animal work described in this study was reviewed by and conducted under the oversight of the University of Texas Southwestern Medical Center Institutional Animal Care and Use Committee and conformed to the National Research Council’s Guide for the Care and Use of Laboratory Animals when establishing animal research standards.

Endotoxemia was induced in young (10-week) and aged (24-week) male mice by lipopolysaccharide (lipopolysaccharide). Based on published results as well as observations in our laboratory, male and female mice showed significantly different susceptibility to systemic symptoms in sepsis models.24 Thus, male but not female mice were chosen for the experiments presented in this report. Lipopolysaccharide was administered intraperitoneally, and mice were weighed individually to determine the exact amount of lipopolysaccharide (MilliporeSigma, Burlington, MA; catalog number L3012) required to achieve the doses indicated in the figure legends. Sterile endotoxin-free PBS was used as a vehicle control in sham groups. In some experiments, TB-peptide, synthesized according to a published sequence25 by NonoPep (Shanghai, China), was administered intraperitoneally at a dose of 16 mg/kg in 100 µL of PBS 30 minutes post lipopolysaccharide challenge.

Echocardiography

Transthoracic echocardiograms were recorded in sedated mice using Visualsonics Vevo 2100 small animal echocardiography machine. Views were taken in planes that approximated the parasternal short-axis view and the apical long-axis view in humans. The cardiac systolic and diastolic functions of randomly selected animals from each group were assessed using the previously described protocol.18,26,27

Preparation of Serum and Tissue Lysates

When animals were euthanized, blood was collected using BD vacutainer rapid serum tubes (BD Diagnostics, Franklin Lakes, NJ) followed by immediate centrifugation at 3000g for 15 minutes at 4 °C to isolate serum. The serum preparations were then allocated and stored at −80 °C until used. Tissues were harvested, washed in PBS, snap clamp frozen, and kept at −80 °C. Tissue lysates were prepared using tissue protein extraction reagent (Thermo Fisher Scientific, Rockford, IL; catalog number 78510). Protein concentrations were quantified using detergent compatible Bradford assay kit (Thermo Fisher Scientific, Rockford, IL; catalog number 23246).

Measurements of Cytokines by ELISA

Cytokine levels in serum were measured using Bio-Plex Mouse Cytokine Panel A 6-Plex (Bio-Rad, Hercules, CA; catalog number M6000007NY) according to vendor’s instructions. Results were normalized by volume of serum samples or by the amount of protein in tissue lysate samples.

Measurement of Myocardial Lactate

The levels of lactate in heart tissue lysates were quantified by lactate assay kit (MilliporeSigma; Catalog Number MAK064) according to vendor’s instructions. Results were normalized by the amount of protein in tissue lysate samples.

Western Blots

Procedures were performed according to established protocol.18 Briefly, prepared SDS-PAGE protein samples were loaded to and run on 15% SDS-PAGE gels and transferred to polyvinylidene fluoride membranes. Membranes were blocked with 5% nonfat milk-PBS at room temperature for 1 hour and subsequently probed with antibody against LC3A/B (Cell Signaling, Danvers, MA; catalog number 4108). The membranes were then rinsed and incubated with corresponding horseradish peroxidase-conjugated antirabbit IgG (Bio-Rad, Hercules, CA; catalog number 170-6515). Antibody dilutions and incubation time were according to manufacturer’s instructions. At the end, membranes were rinsed, and bound antibodies were detected by using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific; catalog number 34077).

Targeted Liquid Chromatography–Mass Spectrometry Metabolite Analysis

(1) Sample preparation: aqueous metabolites were extracted using a methanol-based protein precipitation method as described previously.28 Briefly, heart tissue samples were homogenized in cold water using zirconium oxide beads, methanol was added, and samples were vortexed and then stored for 30 minutes at −20 °C. Afterwards, samples were first sonicated in an ice bath for 10 minutes, centrifuged for 15 minutes at 18,000g and 4 °C, and then a fixed volume of supernatant was collected from each sample. Lastly, recovered supernatants were dried on a SpeedVac and reconstituted for liquid chromatography–mass spectrometry (LC–MS) analysis. Protein pellets that were left over from the sample prep were saved for bicinechonic acid assay. (2) LC–MS analysis: samples were analyzed on a duplex-LC–MS system composed of 2 Shimadzu UPLC pumps, CTC Analytics PAL HTC-xt temperature-controlled auto-sampler, and AB Sciex 6500+ Triple Quadrupole MS equipped
with electrospray ionization source. UPLC pumps were connected to the auto-sampler in parallel and were able to perform 2 chromatography separations independently from each other. Each sample was injected twice on 2 identical analytical columns (Waters XBridge BEH Amide XP) performing separations in hydrophilic interaction liquid chromatography mode. While one column was performing separation and MS data acquisition in electrospray (+) ionization mode, the other column was getting equilibrated for sample injection, chromatography separation and MS data acquisition in electrospray (−) ionization mode. Each chromatography separation was 18 minutes (total analysis time per sample was 36 minutes). (3) Data acquisition: MS data acquisition was performed in multiple-reaction-monitoring mode. The whole LC–MS system was controlled using AB Sciex Analyst 1.6.3 software. Measured MS peaks were integrated using AB Sciex MultiQuant 3.0.3 software. In addition to the study samples, 2 sets of quality control (QC) samples were used to monitor the assay performance as well as data reproducibility. One QC was a pooled human serum sample used to monitor system performance and the other QC was pooled study samples and this QC was used to monitor data reproducibility. Isotope labeled compounds were used to monitor sample preparation and injection. Highly reproducible MS data were generated, having an average coefficient of variance among the metabolites of 5.6%. Data for each sample were normalized according to bicinchoninic acid-based quantification of total protein count.

Statistical Analysis
Analysis was carried out using R (version 4.0.2). The targeted metabolomics assay was designed to detect 361 metabolites and was conducted at the University of Washington’s Nathan Shock Center of Excellence in the Biology of Aging and Northwest Metabolomics Research Center. A median normalization was performed to adjust the data so that samples had the same median value of the metabolite abundance post log2 transformation. Only metabolites with <20% missingness and a coefficient of variance <20% in the pooled sample QC data were considered in further analysis. Out of the possible 361 metabolites that the assay could detect, 161 metabolites passed these filtering criteria, which were included in the imputation step. We used a quantile regression approach for the imputation of left-censored missing data, which has been suggested as the favored imputation method for left-censored missing not at random data. This was implemented in the R imputeLCMD package.

A linear model fit to the normalized metabolomic data using the Bioconductor limma package was used to examine the treatment group difference within the same age group. The limma package uses empirical Bayes moderated statistics, which improves power by “borrowing strength” between metabolites to moderate the residual variance. Metabolites with a false discovery rate of 10% were selected. Two-way or 3-way Venn diagrams were generated to identify common and unique metabolites among comparisons. Pathway analysis was performed by using Shiny GAM (integrated analysis of genes and metabolites) and Cytoscape software. Signaling networks were built on pathway clustering against the small molecule pathway database using MBRole 2.0 software.

RESULTS
TB-Peptide Provides Cardiac Protection in Both Young Adult and Aged Mice During Endotoxemia
Our previous research provided evidence that stimulating Beclin-1 dependent autophagy improves cardiac performance during endotoxemia in young adult mice, and thus TB-peptide holds a promising therapeutic potential for sepsis. In this report, we examined whether TB-peptide exerts a similar protective effect on aged animals under the same condition. In our experimental setting, sham or lipopolysaccharide challenge was administered to groups of 24-month-old and 10-week-old mice at indicated dosages, followed by treatment with TB-peptide, and echocardiography was used to assess heart performance.

Consistent with literature and as expected, we observed that older mice were more susceptible to the toxic effects induced by lipopolysaccharide. The 24-month-old (aged) mice showed impaired cardiac function but were able to survive when receiving lipopolysaccharide challenged at 1 mg/kg. However, greater fatality was observed when lipopolysaccharide dose was increased to 3 mg/kg. In 10-week-old (young adult) mice, 3 mg/kg lipopolysaccharide triggered heart dysfunction without impact on survival, whereases at 10 mg/kg, we observed significant lipopolysaccharide-induced fatality in the group. Because of the different sensitivities to lipopolysaccharide between the aged and young adult mice, we were not able to choose a universal dose of lipopolysaccharide to induce cardiac dysfunction and to perform follow-up analysis in both groups. Therefore, we used the physiological function of the heart as a base for comparison in the studies performed in this report.

As shown in Figure 1A and 1B, at the levels of lipopolysaccharide challenge that inducing significant reduction in cardiac contractility in young or aged mice, administration of TB-peptide was able to rescue cardiac performance, demonstrated by its improvement in fractional shortening and ejection fraction. Further,
Figure 1. Cardiac protective effects of TB-peptide in young adult and aged mice during endotoxemia. Mice were given 5 mg/kg lipopolysaccharide intraperitoneally and TB-peptide, 16 mg/kg, was administered intraperitoneally 30 minutes post lipopolysaccharide challenge. Experiments were performed 18 hours post challenge. Cardiac function was evaluated by echocardiography in the young adult (A, 5/group) and aged (B, 6/group) mice. Circulating cytokine levels were measured in blood serum prepared from the young adult (C, 5/group) and aged (D, 5/group) groups by ELISA. In harvested heart tissue, autophagy marker LC3II was detected by Western blot using the total tissue lysates, and signals were quantified by densitometry (E, 5/group). Levels of lactate were quantified in the heart tissue lysates (F, 5/group). All data were expressed as mean±SEM of at least 3 independent experiments. Data were analyzed by 2-way ANOVA with post hoc test for comparisons of multiple groups and Student t test for comparisons between 2 groups using GraphPad Prism software. Differences were considered statistically significant as P≤0.05. Significant differences are shown as * for sham vs lipopolysaccharide and ** for with vs without the treatment of TB-peptide (A through E) or for difference between age groups (F). IFN indicates interferon; IL, interleukin; LPS, lipopolysaccharide; and TNFα, tumor necrosis factor alpha.
TB-peptide-mediated reduction in inflammation was demonstrated by its attenuation of circulating cytokines (Figure 1C and 1D). Consistent with published results in the literature from us and others, we confirmed that this TB-peptide was able to boost cardiac autophagy response in both young and old animals during...
endotoxemia, shown by enhanced signal of LC3II in the heart tissue lysates (Figure 1E). Because lactate is a metabolic intermediate mediates both glucose metabolism and fatty acid metabolism, we measured levels of lactate in the heart tissue of 10-week-old and 24-month-old mice for the purpose of testing whether

Figure 1. Continued
cardiac performance associates with myocardial metabolic changes. We observed that lipopolysaccharide challenge produced a significant increase in lactate in young mice but not in old mice (Figure 1F), suggesting that lipopolysaccharide-stimulated shifting of cardiac energy metabolism is at least partially affected by aging.

A Targeted Metabolomics Study to Compare Myocardial Metabolite Profiling in Response to Endotoxemia and to the Follow-Up Therapeutic Treatment by TB-Peptide Between Young Adult and Aged Mice

LC–MS metabolomics analysis was applied to the heart tissue samples harvested from the experimental groups of young and aged mice subjected to lipopolysaccharide challenge or sham followed by treatment with TB-peptide or vehicle (Table 1).

A targeted approach was chosen, in which profiling covers 361 known metabolites that were selected based on published results showing their association with over 50 regulatory pathways in almost all aspects of myocardial metabolisms, such as central carbon metabolism (glycolysis tricarboxylic acid cycle, pentose phosphate), AA metabolism (branched-chain AAs, urea cycle), lipid metabolism (choline, fatty acids), and purine metabolism. Evaluation of data quality, exploratory analysis, and data preprocessing are summarized in Data S1. Across a total of 43 mouse heart samples, 156 metabolites were measured with detectable abundance, having missingness <20% and coefficient of variance <20% by univariate analysis. In the comparisons between groups, changes in metabolites showing false discovery rate of 10% or less were considered having statistical significance. As summarized in Table 2, lipopolysaccharide induced similar levels of significant changes in the number of metabolites in young and aged groups, 69 versus 62 respectively. In groups receiving TB-peptide, lipopolysaccharide altered levels of 42 metabolites in the young mice versus 60 in the aged mice. When under endotoxemia, TB-peptide altered 30 metabolites in the young versus 11 in the old mice. As expected, the peptide changed little or none in the sham controls of both young and aged groups. Analysis and comparisons of changes in metabolite profiles induced by endotoxemia and by TB-peptide in young and old mice are described in detail in the following sections.

Myocardial Metabolite Profiling in Response to Endotoxemia and to the Follow-Up Therapeutic Treatment With TB-Peptide in Young Adult Mice

We first compared the metabolic profiles in the heart of young adult mice (10 weeks old) challenged with lipopolysaccharide or sham and their responses to the treatment with TB-peptide. Figure 2A summarized the interactive mean-difference plots of 4 comparison groups, including lipopolysaccharide-treated versus sham, lipopolysaccharide-challenged versus sham under the treatment of TB-peptide, sham group with peptide treatment versus untreated, and lipopolysaccharide group with peptide treatment versus untreated. Names of these metabolites, together with their values of log2-fold change (FC), average log2-abundance, and false discovery rate, are listed in Tables 3 through 5. Among the 156 targets with detectable significance, lipopolysaccharide challenge caused increases in 50 and decreases in 19 metabolites (Table 3). N-acetylglycine, a derivative of AA glycine metabolism, was shown the most increased metabolite with a 5.6-fold increase in response to lipopolysaccharide. Adenosine, whose derivatives function as energy carriers in forms of AMP, ADP, and ATP, was identified as the most significantly decreased metabolite with a change of over

Table 1. List of Animal Numbers Tested in Each Group

| Age          | Endotoxemia | Treatment | Number |
|--------------|-------------|-----------|--------|
| Aged 24-mo   |             | None      | 5      |
|              | Lipopolysaccharide | 5        |
|              | Sham        | TB-peptide | 4      |
|              | Lipopolysaccharide | 5        |
| Young 10-wk  |             | None      | 6      |
|              | Lipopolysaccharide | 6        |
|              | Sham        | TB-peptide | 6      |
|              | Lipopolysaccharide | 6        |

Table 2. List of Numbers of Metabolites With Statistically Significant Changes

| Experimental groups | Comparisons | Metabolites with changes in significance (false discovery rate <0.1) |
|---------------------|-------------|---------------------------------------------------------------------|
| Young 10-wk          | Lipopolysaccharide vs sham | 69                                                                    |
|                     | Lipopolysaccharide vs sham under TB-peptide | 42                                                                  |
|                     | With vs without TB-peptide in shams | 0                                                                  |
|                     | With vs without TB-peptide in lipopolysaccharide challenged | 30                                                                |
| Aged 24-mo           | Lipopolysaccharide vs sham | 62                                                                    |
|                     | Lipopolysaccharide vs sham under TB-peptide | 60                                                                  |
|                     | With vs without TB-peptide in shams | 1                                                                  |
|                     | With vs without TB-peptide in lipopolysaccharide challenged | 11                                                                  |
11-fold by lipopolysaccharide. As listed in Table 4, treatment with TB-peptide reduced the scope of lipopolysaccharide-induced changes in metabolites, which included upregulation in 28 and downregulation in 14 metabolites. The treatment also decreased the levels of changes. For example, lipopolysaccharide-triggered fold changes in N-acetyl-glycine and adenosine were reduced to 2.86 and 4.14 respectively, compared with 5.6 and 11 when TB-peptide was not given. However, in the case of UDP-glucose, an intermediate of synthesis of glycogen, lipopolysaccharides, and glycosphingolipids, the fold change of downregulation was increased from 7.9 to 12.67. As expected, TB-peptide did not incite detectable changes in sham animals, whereas the treatment increased the abundance in 8 but reduced in 22 metabolites in lipopolysaccharide-challenged groups (Table 5).

To further investigate the impact of TB-peptide in myocardial metabolites during endotoxemia, we compared the TB-peptide treated groups of sham and lipopolysaccharide-challenged mice with those without the treatment. As shown in Figure 2B, 31 metabolites...
were identified as having lipopolysaccharide-associated changes regardless of the presence of TB-peptide. However, in more than half of these metabolites, lipopolysaccharide-associated fold changes were attenuated by TB-peptide, for example, in adenosine and N-acetyl-glycine. Additionally, lipopolysaccharide altered the abundance in 38 metabolites, which were not affected by TB-peptide. On the other hand, when

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| Metabolite                        | Log2 Fold Change | Log2 Fold Change |
|-----------------------------------|------------------|------------------|
| isoValerylcarbamide               | -2               | 3-Indoxyl Sulfate|
| Methionine Sulfoxide              | -1               | Trigonelline     |
| Asparagine                        | 0                | Citrulline       |
| Orotate                           | 1                | Histidine        |
| S-Methylcysteine                  | 2                | iso-Leucine/allo-leucine |
| Valine                            | -2               | Tryptophan       |
| betaAlanine                       | -1               | Dimethylarginine (A/SDMA) |
| Adenylosuccinate                  | 0                | Argininosuccinate|
| Thiamine                          | 1                | Oxalacetate      |
| Linoelic Acid                     | 2                | 3-Methyl-3-Hydroxyglutaric Acid |
| Phosphocreatine                   | -2               |                  |
| 1-Methylnicotinamide              | -1               |                  |
| NAD                               | 0                |                  |
| trans-Aconitate                   | 1                |                  |
| Carnosine                         | 2                |                  |
| Dimethylglycine                   | -2               |                  |
| N-Carbamoyl-B-Alanine             | -1               |                  |
| N-isoValerylglycine               | 0                |                  |
| 7-Methylguanidine                 | 1                |                  |

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**Figure 2.** Continued
receiving TB-peptide treatment, lipopolysaccharide stimulated changes in 11 metabolites, which differences were not detectable in the absence of the peptide.

Using the data of metabolic changes summarized above, we performed pathway analysis using Shiny GAM and Cytoscape software and signaling network analysis based on the small molecule pathway database. Results showed that lipopolysaccharide significantly impaired pathways of carbohydrate/glucose metabolism and AA metabolism, including the malate–aspartate shuttle, D-glutamine/D-glutamate transition, alanine-aspartate–glutamate cycling, arginine-proline synthesis, glycine-serine–threonine pathway, and purine metabolism. In the meantime, lipopolysaccharide upregulated fatty acid metabolism, such as glycolipids and linoleic acid (Figure 2C). With the treatment of TB-peptide, the metabolism via AAs and glucose pathways was significantly improved whereas elevation in
**Table 3. Lipopolysaccharide-Induced Significant Changes in Myocardial Metabolites of Young Mice**

| Metabolite                  | HMDB.ID   | KEGG.ID   | logFC  | log2 abundance | FDR            |
|-----------------------------|-----------|-----------|--------|----------------|----------------|
| Adenosine                   | HMDB00050 | C00212    | −3.50  | 23.86         | 4.54E-06       |
| UDP-glucose                 | HMDB00286 | C00029    | −2.98  | 17.63         | 4.86E-05       |
| isoValeryl carnitine        | HMDB0688  | C20826    | −2.48  | 18.63         | 4.86E-05       |
| Adenine                     | HMDB00334 | C00147    | −2.27  | 21.22         | 1.47E-06       |
| Glycerophosphocholine       | HMDB0086  | C00670    | −1.93  | 23.04         | 2.19E-07       |
| Acetylcarnitine             | HMDB00201 | C02571    | −1.79  | 25.25         | 0.0052         |
| Methionine                  | HMDB0696  | C00073    | −1.38  | 18.29         | 3.94E-06       |
| Methionine sulfoxide        | HMDB02005 | C02989    | −1.31  | 15.29         | 0.0098         |
| Serine                      | HMDB00187 | C00065    | −1.13  | 21.34         | 3.63E-05       |
| Pentothenate                | HMDB00210 | C00864    | −1.05  | 22.35         | 0.0043         |
| Asparagine                  | HMDB00168 | C00152    | −0.87  | 19.14         | 0.0003         |
| Oxidized glutathione        | HMDB03337 | C00127    | −0.69  | 21.89         | 0.0123         |
| Hypoxanthine                | HMDB00157 | C00262    | −0.62  | 25.19         | 0.0049         |
| Arabitol/xylitol            | HMDB00568 | C01904    | −0.62  | 17.20         | 0.0131         |
| Guanosine                   | HMDB00133 | C00387    | −0.65  | 19.31         | 0.0151         |
| Aspartic acid               | HMDB00191 | C00049    | −0.61  | 22.58         | 0.0306         |
| Orotate                     | HMDB00226 | C00296    | −0.54  | 15.43         | 0.0519         |
| Tyrosine                    | HMDB00158 | C00082    | −0.39  | 20.34         | 0.0437         |
| S-methylcysteine            | HMDB02108 | C20404    | −0.33  | 17.81         | 0.0945         |
| Phenylalanine               | HMDB00159 | C00079    | 0.38   | 22.24         | 0.0133         |
| o-phosphoethanolamine       | HMDB000224| C00346    | 0.44   | 22.96         | 0.0146         |
| Arachidonate                | HMDB06102 | C00219    | 0.45   | 22.58         | 0.0306         |
| Ribulose 5-phosphate        | HMDB00618 | C00199    | 0.49   | 21.67         | 0.0973         |
| Valine                      | HMDB00883 | C00183    | 0.50   | 19.00         | 0.0150         |
| Ethanolamine                | HMDB00149 | C00189    | 0.58   | 15.45         | 0.0149         |
| N-Ac-alanine                | HMDB00766 |          | 0.59   | 17.85         | 0.0018         |
| betaAlanine                 | HMDB00056 | C00099    | 0.60   | 15.55         | 0.0772         |
| Riboflavin                  | HMDB00244 | C00255    | 0.62   | 17.08         | 0.0005         |
| Anserine                    | HMDB00194 | C01262    | 0.64   | 18.90         | 0.0115         |
| Adenylosuccinate            | HMDB00536 | C03794    | 0.65   | 17.17         | 0.0437         |
| 2-Hydroxyphenylacetate      | HMDB06235 | C01983    | 0.65   | 14.77         | 0.0133         |
| Thiamine                    | HMDB00235 | C00378    | 0.66   | 19.02         | 0.0501         |
| N2, N2-dimethylguanosine    | HMDB04824 | C06492    | 0.66   | 13.74         | 0.0039         |
| Linoleic acid               | HMDB00673 | C01595    | 0.68   | 20.16         | 0.0020         |
| 1/3-methylhistidine         | HMDB00001 | C01152    | 0.68   | 17.57         | 0.0136         |
| Uridine                     | HMDB00296 | C00299    | 0.69   | 19.69         | 0.0026         |
| Phosphocreatine             | HMDB01311 | C02305    | 0.70   | 16.42         | 0.0273         |
| N6-trimethyllysine          | HMDB01325 | C03793    | 0.72   | 20.39         | 0.0193         |
| Cystathionine               | HMDB00099 | C02291    | 0.72   | 14.25         | 0.0306         |
| 1-Methylnicotinamide        | HMDB00699 | C02918    | 0.73   | 17.65         | 0.0181         |
| Glycerol-3-P                | HMDB00126 | C00063    | 0.77   | 24.48         | 0.0271         |
| Lactose/trehalose           | HMDB00186 | C00243    | 0.79   | 18.99         | 0.0434         |
| NAD                         | HMDB00902 | C00003    | 0.84   | 17.38         | 0.0061         |
| IMP                         | HMDB00175 | C00130    | 0.85   | 26.20         | 0.0019         |
| trans-Aconitate             | HMDB00958 | C02341    | 0.85   | 13.41         | 0.0784         |
| Homocitrulline              | HMDB00679 | C02427    | 0.93   | 15.35         | 0.0306         |
| NADP                        | HMDB00217 | C00006    | 0.95   | 12.74         | 0.0924         |
| Carnosine                   | HMDB00333 | C00386    | 1.05   | 21.21         | 0.0002         |

(Continued)
fatty acid synthesis was attenuated (Figure 2D). These data suggest that the application of TB-peptide was able to rectify the alteration induced by lipopolysaccharide in heart of young adult mice, and thus, ameliorate cardiac function.

Myocardial Metabolite Profiling in Response to Endotoxemia and to the Follow-Up Therapeutic Treatment With TB-Peptide in Aged Mice

Similarly, the metabolic profiles in the hearts of aged mice (24 months old) from sham versus lipopolysaccharide-challenge groups and their responses to TB-peptide treatment were examined. The interactive mean-difference plots of 4 comparisons, including lipopolysaccharide-challenged versus sham, lipopolysaccharide-challenged versus sham under TB-peptide treatment, sham group with peptide treatment versus untreated, and lipopolysaccharide group with peptide treatment versus untreated, were summarized in Figure 3A. Metabolites detected with statistical significance, together with their values of fold change, average log2-abundance, and false discovery rate, are listed in Tables 6 through 8. Of the 156 metabolites, 24 targets were significantly elevated and 38 decreased by challenge with lipopolysaccharide (Table 6). Among these molecules, allantoin, the main product of uric acid oxidation, was increased the most, with a fold change of about 4.8, by lipopolysaccharide. On the other hand, as in the young mice, adenosine was identified as the most downregulated metabolite by lipopolysaccharide with a fold change of 3.2 in the heart of aged mice. In animals receiving TB-peptide, lipopolysaccharide triggered significant increases in 18 and decreases in 42 metabolites in the heart (Table 7). Under this condition, N-carbamoyl-β-alanine, a urea derivative of β-alanine, was the most upregulated metabolite with a fold-difference of 3.8, compared with the unchallenged sham controls. Lipopolysaccharide stimulated allantoin, but the fold difference was reduced to 3.3 by TB-peptide from 4.8 in the absence of the peptide treatment. Methionine sulfoxide, the oxidized form of methionine and a marker of oxidative stress, was found to be downregulated the most with a change of 2.5-fold. Interestingly, lipopolysaccharide-associated decrease in adenosine decrease was undetectable under the treatment of TB-peptide, suggesting a TB-peptide-mediated effect of improving energy production.

### Table 3. Continued

| Metabolite                | HMDB.ID  | KEGG.ID | logFC   | log2 abundance | FDR          |
|---------------------------|----------|---------|---------|----------------|--------------|
| Glucosamine-6-phosphate   | HMDB0001254 | C00352 | 1.130319574 | 16.1179439    | 0.01520334   |
| Sedoheptulose 7-phosphate | HMDB01068 | C05382 | 1.132445025 | 21.99544352   | 0.00058224   |
| 3-Hydroxyisovaleric acid  | HMDB00754 | C20827 | 1.1786381  | 16.50883966   | 7.8683E-05   |
| Dimethylglycine           | HMDB00092 | C01026 | 1.27381044  | 16.24965502   | 0.000174352  |
| Uric acid                 | HMDB00300 | C00106 | 1.290287028 | 20.86123826   | 2.4256E-10   |
| Cytidine                  | HMDB00089 | C00475 | 1.406732689 | 21.73604314   | 2.1474E-08   |
| 2’-Deoxycytidine          | HMDB00014 | C00881 | 1.407228732 | 17.49697137   | 4.248E-09    |
| 2-Aminoadipate            | HMDB00510 | C00956 | 1.41470616  | 16.87943741   | 0.001028555  |
| 1-Methyladenosine         | HMDB00331 | C02494 | 1.454698084 | 16.60747522   | 1.9994E-06   |
| N-carbamoyl-β-alanine     | HMDB00026 | C02642 | 1.520205652 | 14.5796584    | 0.024131976  |
| Allantoin                 | HMDB00462 | C01551 | 1.647463659 | 20.63393545   | 0.000563337  |
| Glutaryl carnitine        | HMDB13130 | C02494 | 1.807288491 | 17.65586613   | 1.8517E-05   |
| n-valerylglycine          | HMDB00678 | C02494 | 1.854144069 | 15.57962302   | 0.00420179   |
| 3HBA                      | HMDB0000357 | C01089 | 1.871240980 | 21.08722141   | 0.000162793  |
| 2-Hydroxyisobutyrate/2-   | HMDB00729 | C01089 | 1.941624661 | 18.70370022   | 2.1996E-07   |
| hydroxybutyrate            |           |         |         |                |              |
| Succinyl carnitine         | HMDB61717 | C01089 | 1.957087967 | 23.05988206   | 2.1474E-08   |
| G6P                       | HMDB0001401 | C00902 | 2.126089231 | 26.42552439   | 0.000268097  |
| 7-Methylguanine           | HMDB00087 | C02242 | 2.184038818 | 15.9037898    | 2.824E-05    |
| Pseudouridine              | HMDB00767 | C02067 | 2.241822376 | 19.02450924   | 1.9771E-05   |
| G1P/F1P/F6P                | HMDB05186 | C01094 | 2.305984672 | 24.44804743   | 0.00017851   |
| N-AcetylGlycine            | HMDB00352 | C0103 | 2.48696726  | 16.97801982   | 2.4256E-10   |

3HBA, 3-hydroxybutyric acid; FC indicates fold change; FDR, false discovery rate; G6P, glucose 6-phosphate; IMP, inosine monophosphate; NAD, nicotinamide adenine dinucleotide; NADP, nicotinamide adenine dinucleotide phosphate; and UDP, uridine diphosphate.
### Table 4. Lipopolysaccharide-Induced Significant Changes in Myocardial Metabolites of Young Mice Receiving TB-Peptide

| Metabolite                        | HMDB.ID       | KEGG.ID   | logFC  | log2 abundance | FDR             |
|-----------------------------------|---------------|-----------|--------|----------------|-----------------|
| UDP-glucose                       | HMDB00286     | C00029    | -3.663516753 | 17.63404042 | 1.11579E-05     |
| Acetyl carnitine                  | HMDB00201     | C02571    | -2.051778059 | 25.2547884  | 0.000247391     |
| Adenosine                         | HMDB00050     | C00212    | -1.917670301 | 23.86931938 | 0.014122908     |
| 3-indoxyl sulfate                 | HMDB00682     | C01004    | -1.70357922  | 17.91025929 | 0.026552223     |
| Trigonelline                      | HMDB00875     | C01904    | -1.60698519  | 17.87022517 | 4.36735E-05     |
| Adenine                           | HMDB00034     | C00147    | -1.262324222 | 21.22184279 | 0.018246811     |
| Araarbol/xylitol                  | HMDB00568     | C00073    | -0.935467711 | 18.29150982 | 0.00168457      |
| Methionine                        | HMDB00696     | C00079    | -0.50558362  | 21.03427896 | 0.08018539      |
| Guanosine                         | HMDB00133     | C00387    | -0.837615621 | 19.31073305 | 0.016966526     |
| Citrulline                        | HMDB00904     | C00327    | -0.696056936 | 22.13035679 | 0.018246811     |
| Hypoxanthine                      | HMDB00157     | C00262    | -0.604536334 | 25.19458686 | 0.003084473     |
| Oxidized glutathione              | HMDB00337     | C0127     | -0.538028531 | 21.8909802  | 0.077134029     |
| Serine                            | HMDB00187     | C00065    | -0.50558362  | 21.03427896 | 0.08018539      |
| Histidine                         | HMDB00177     | C00135    | -0.400626334 | 22.92866174 | 0.045506999     |
| Phenylalanine                     | HMDB00159     | C00079    | 0.360855597  | 22.2348415  | 0.031563198     |
| iso-Leucine/allo-isoleucine       | HMDB00172/   | C04077    | 0.407011027  | 18.72100738 | 0.045506999     |
| Dimethylarginine (A/SDMA)         | HMDB00139/    | C03626    | 0.598466485  | 18.23516339 | 0.053269395     |
| Argininosuccinate                 | HMDB00052     | C03406    | 0.629467478  | 17.15747824 | 0.064830017     |
| Ribulose 5-phosphate              | HMDB00061     | C00199    | 0.632741879  | 21.6725341 | 0.039872264     |
| N6-Trimethyllysine                | HMDB00135     | C03793    | 0.681343752  | 20.39412577 | 0.045506999     |
| 1/3-Methylhistidine               | HMDB00001     | C01152    | 0.733773991  | 17.57160604 | 0.016966526     |
| Oxalaceta                         | HMDB00223     | C00036    | 0.772924705  | 13.71753688 | 0.012311173     |
| 2′-Deoxyctydine                   | HMDB00014     | C00881    | 0.789242017  | 17.94961737 | 0.000247391     |
| Glutaricarnitine                  | HMDB01330     | C00079    | 0.79820565  | 17.56586613 | 0.068927058     |
| Uracil                            | HMDB00300     | C00106    | 0.81276773  | 20.86123826 | 1.11579E-05     |
| Sedoheptulose 7-phosphate         | HMDB01068     | C05382    | 0.830566254  | 21.9954532  | 0.020701305     |
| Homocitrulline                    | HMDB00079     | C00427    | 0.831176457  | 15.35024408 | 0.085314121     |
| Cytidine                          | HMDB00089     | C00475    | 0.871603261  | 21.73604314 | 0.000247391     |
| Xanthosine                        | HMDB00299     | C01762    | 0.887764943  | 16.32577761 | 0.007986437     |
| 3-Methyl-3-hydroxyglutaric acid   | HMDB0000355   | C03761    | 0.91046067  | 12.6291266  | 0.030332094     |
| Lactose/trehalose                 | HMDB00186     | C00243    | 0.96657408  | 18.9809587 | 0.02591865      |
| Glucosamine-6-phosphate           | HMDB0001254   | C00352    | 1.163982793  | 16.1179439  | 0.023982594      |
| 2-Aminoadipate                    | HMDB00510     | C00956    | 1.47129853  | 16.87943741 | 0.001561702     |
| N-AcetylGlycine                   | HMDB00532     | 1.516513784 | 16.97801982 | 2.27301E-05  |
| 2-Hydroxyisobutyrate/2-           | HMDB00729     | 1.533744624 | 18.70370022 | 4.36735E-05  |
| hydroxybutyrate                   | HMDB00185     | C01094    | 2.378040124  | 24.44804743 | 0.000247391     |
| G1P/F1P/F6P                       | HMDB001076    | C00085    | 2.42346041  | 26.42552439 | 0.000141886     |

FC indicates fold change; FDR, false discovery rate; UDP, uridine diphosphate.
There was little effect of TB-peptide on cardiac metabolites in sham control animals; the only molecule with significant change was guanidinoacetate, showing a decrease of 2.3-fold (Table 8). In animals challenged by lipopolysaccharide, TB-peptide treatment led to decreases in 10 and an increase in 1 metabolite in aged hearts (Table 8).

The effects of TB-peptide on myocardial metabolites in aged mice during endotoxemia were also analyzed by comparing data from the TB-peptide treated groups of sham and lipopolysaccharide-challenged mice with those from animals without the treatment. As summarized in Figure 3B, 37 metabolites were identified having lipopolysaccharide-associated changes regardless of the presence of TB-peptide. However, in 18 of these molecules, lipopolysaccharide-induced fold changes were attenuated by TB-peptide. In addition, 25 metabolites that were altered by lipopolysaccharide but had little response to TB-peptide. Further, in animals given TB-peptide treatment, lipopolysaccharide altered 23 new metabolites compared with the condition of without the peptide treatment.

Metabolic profiling from the aged mice was applied to pathway analysis and network analysis as described previously. Results suggest that metabolites in pathways of glucose and amino acid metabolism were significantly downregulated in aged heart by endotoxemia (Figure 3C). Treatment with TB-peptide reversed the responses of these pathways (Figure 3D), consistent with the observations obtained in the young hearts.

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### Table 5. TB-Peptide-Induced Significant Changes in Myocardial Metabolites of Young Mice

| Metabolite                                      | HMDB.ID | KEGG.ID | logFC | log2 abundance | FDR       |
|------------------------------------------------|---------|---------|-------|----------------|-----------|
| Lipopolysaccharide-challenged group             |         |         |       |                |           |
| n-isovalerylglycine                             | HMDB00678 |       | -2.960102541 | 15.97982302 | 8.8989E-05 |
| 7-Methylguanine                                 | HMDB00897 | C02242  | -1.967084102 | 15.09317898 | 0.000298167 |
| Pseudouridine                                   | HMDB00767 | C02067  | -1.564571918 | 19.02450924 | 0.003936741 |
| N-AcetylGlycine                                 | HMDB00532 |       | -1.285854526 | 16.97801982 | 0.000257584 |
| 1-Methylnicotinamide                            | HMDB00699 | C02918  | -1.226467546 | 17.65160318 | 0.000298167 |
| Succinylcarnitine                               | HMDB61717 |       | -1.214433733 | 23.05998206 | 0.000297102 |
| 1-Methyladenosine                               | HMDB00331 | C02494  | -1.192026095 | 16.60747522 | 0.000170922 |
| Trigonelline                                    | HMDB00875 | C01004  | -1.106743314 | 17.87022517 | 0.003936741 |
| 3-Hydroxyisovaleric acid                       | HMDB00754 | C02087  | -1.071695407 | 16.5083966 | 0.000594075 |
| Phosphocreatine                                 | HMDB01511 | C02305  | -0.916614511 | 16.42700403 | 0.009986574 |
| Glycerol-3-P                                    | HMDB00126 | C00093  | -0.884612321 | 24.48432725 | 0.025786544 |
| Dimethylglycine                                 | HMDB00092 | C01026  | -0.873442773 | 16.24965502 | 0.01893279 |
| Suberic acid                                    | HMDB00893 | C08278  | -0.856700702 | 15.9663203 | 0.097925558 |
| beta-Alanine                                    | HMDB00058 | C00099  | -0.809115183 | 15.55146758 | 0.031939882 |
| Uridine                                        | HMDB00296 | C00299  | -0.548756032 | 19.69582063 | 0.031939882 |
| N2, N2-Dimethylguanosine                       | HMDB04824 |         | -0.538285701 | 13.74037868 | 0.031939882 |
| 2’-Deoxyctydine                                 | HMDB00014 | C00881  | -0.511896569 | 17.49697137 | 0.02535051 |
| Cytidine                                       | HMDB00089 | C00475  | -0.504341571 | 21.73604314 | 0.03623811 |
| Linoleic acid                                   | HMDB00673 | C01595  | -0.486995048 | 20.1608533 | 0.05098851 |
| N-Ac-alanine                                    | HMDB00766 |         | -0.485510673 | 17.8530671 | 0.003936741 |
| Uracil                                         | HMDB00200 | C00106  | -0.45409582  | 20.86123826 | 0.009879525 |
| Palmitic acid                                   | HMDB000220| C00249  | -0.41652477 | 18.77132958 | 0.096541724 |
| Methionine                                      | HMDB00696 | C00073  | 0.660600291 | 18.29150982 | 0.031939882 |
| Lysine                                          | HMDB00182 | C00047  | 0.67590058  | 22.74394835 | 0.00672983 |
| Argininosuccinate                               | HMDB00052 | C03408  | 0.764288611 | 17.15747824 | 0.00672983 |
| Serine                                          | HMDB00187 | C00065  | 0.844245287 | 21.03427896 | 0.003529375 |
| Arginine                                        | HMDB00517 | C00062  | 0.86584688  | 22.91032246 | 0.040308837 |
| Asparagine                                      | HMDB00168 | C00152  | 0.978183481 | 19.14028377 | 0.000298167 |
| Pentothenate                                    | HMDB00210 | C00864  | 1.661246962 | 22.34537961 | 8.8989E-05 |
| Glycero phosphocholine                          | HMDB00086 | C00670  | 1.888270369 | 23.04142803 | 2.39539E-06 |
| Sham group                                      |         |         |       |                |           |
| None                                           | N/A     | N/A     | N/A   | N/A            | N/A       |

FC indicates fold change; and FDR, false discovery rate.
However, unlike the young counterparts, lipopolysaccharide induced a decrease in fatty acid metabolism, and TB-peptide had little effect on this response.

**Age-Dependent and -Independent Changes in Myocardial Metabolite Profiling in Response to Endotoxemia and to the Therapeutic Treatment by TB-Peptide**

To address whether age plays an important role in altering myocardial metabolites in response to endotoxemia and to the treatment of TB-peptide, we compared compounds with significant changes between groups of young and old mice with or without lipopolysaccharide challenge and with or without the treatment of TB-peptide. As shown in Figure 4, the heatmap comparison indicates that TB-peptide mitigated lipopolysaccharide-induced impairment in amino acid biosynthesis via glutamate-aspartate pathway in both young and aged groups. A distinct age-dependent pattern was found to associate with metabolites involved in fatty acid metabolism, such as choline, phosphocholine, linolenic acid, linoleic acid, and 1-methylnicotinamide, as well as in AAs that were previously reported to be closely related to fatty acid
Figure 3. Continued

metabolism, such as isoleucine and valine. In this category of molecules, TB-peptide appeared to attenuate lipopolysaccharide-induced changes in the young mice but had moderate or little effect in the aged group.

DISCUSSION

We previously demonstrated that promoting autophagy via Beclin-1 is cardiac protective in a mouse...
model of endotoxemia. Additionally, pharmacological Beclin-1 activator TB-peptide exhibited therapeutic potential in several preclinical models including cancer chemotherapy, infection, endotoxemia, and pneumonia-induced sepsis. In the studies summarized here, we obtained results supporting that
Table 6. Lipopolysaccharide-Induced Significant Changes in Myocardial Metabolites of Old Mice

| Metabolite                     | HMDB.ID   | KEGG.ID  | logFC     | log2 abundance | FDR        |
|--------------------------------|-----------|----------|-----------|----------------|------------|
| Adenosine                      | HMDB00050 | C00212   | -1.688     | 23.869         | 0.034       |
| Guanosine                      | HMDB00133 | C00387   | -1.296     | 19.310         | 0.0005      |
| Homoaarginine                  | HMDB00670 | C01924   | -1.244     | 16.563         | 0.0022      |
| Adenine                        | HMDB00034 | C00147   | -1.193     | 21.222         | 0.0303      |
| Asparagine                     | HMDB00168 | C00152   | -1.164     | 19.140         | 0.0001      |
| Glucose                        | HMDB00122 | C00331   | -1.149     | 17.517         | 0.0003      |
| Acetylcarnitine                | HMDB00201 | C02571   | -1.065     | 25.255         | 0.0692      |
| Serine                         | HMDB00187 | C00065   | -1.056     | 21.034         | 0.0006      |
| Methionine                     | HMDB00696 | C00073   | -1.031     | 18.291         | 0.001       |
| Aspartic acid                  | HMDB00191 | C00049   | -1.026     | 22.258         | 0.001       |
| Argininosuccinate              | HMDB00052 | C03406   | -1.014     | 17.157         | 0.0003      |
| gamma-Aminobutyrate            | HMDB000112| C00334   | -0.982     | 16.108         | 0.0006      |
| Glutamic acid                  | HMDB000148| C00205   | -0.947     | 25.039         | 0.0036      |
| S-adenosylmethionine (SAM)     | HMDB01185 | C00199   | -0.940     | 19.991         | 0.0046      |
| Threonine                      | HMDB00167 | C00188   | -0.920     | 21.053         | 0.0001      |
| Arginine                       | HMDB00517 | C00629   | -0.903     | 22.910         | 0.0001      |
| S-Methylthioadenosine          | HMDB01173 | C00170   | -0.840     | 17.593         | 0.0046      |
| Glycerophosphocholine          | HMDB00086 | C00670   | -0.820     | 23.041         | 0.0026      |
| Inosine                        | HMDB00195 | C00294   | -0.813     | 24.053         | 0.0029      |
| Guanidinoacetate               | HMDB00128 | C00581   | -0.770     | 15.092         | 0.0066      |
| Lysine                         | HMDB00182 | C00047   | -0.753     | 22.743         | 0.0039      |
| Phosphocreatine                | HMDB01511 | C02305   | -0.639     | 16.427         | 0.0080      |
| Hypoxanthine                   | HMDB00157 | C00262   | -0.639     | 25.194         | 0.0033      |
| Choline                        | HMDB00097 | C00114   | -0.630     | 24.401         | 0.0002      |
| Linolenic acid                 | HMDB01388 | C06427   | -0.596     | 15.633         | 0.0052      |
| Glutamine                      | HMDB00641 | C00064   | -0.586     | 26.152         | 0.0051      |
| Guanine                        | HMDB00132 | C00242   | -0.577     | 14.685         | 0.0012      |
| CDP                            | HMDB01546 | C00112   | -0.565     | 16.055         | 0.0070      |
| Pyroglutamic acid              | HMDB00267 | C01879   | -0.555     | 17.167         | 0.0026      |
| N-acetyl-aspartate (NAA)       | HMDB00812 | C01042   | -0.530     | 20.674         | 0.0015      |
| Glycine                        | HMDB00123 | C00037   | -0.524     | 16.908         | 0.0027      |
| Oxidized glutathione           | HMDB00337 | C00127   | -0.520     | 21.890         | 0.0012      |
| CMP                            | HMDB00095 | C00055   | -0.492     | 16.814         | 0.0007      |
| Histidine                      | HMDB00177 | C00135   | -0.460     | 22.928         | 0.0026      |
| Xanthine                       | HMDB00292 | C00385   | -0.460     | 22.758         | 0.0039      |
| Ribose-5-P                     | HMDB01548 | C00117   | -0.449     | 23.262         | 0.0034      |
| Leucine /D-norleucine          | HMDB00687 | C00123   | -0.441     | 21.867         | 0.0044      |
| Tryptophan                     | HMDB00929 | C00078   | -0.417     | 19.193         | 0.0089      |
| isoLeucine/alloisoLeucine      | HMDB00172/| C00407/   | -0.354     | 18.712         | 0.0019      |
| isoLeucine/alloisoLeucine      | HMDB00057 | C21096   | -354.353   | 18.712         | 0.0019      |
| N-Ac-alanine                   | HMDB00766 |               | 0.298      | 17.853         | 0.0089      |
| Uridine                        | HMDB00296 | C00299   | 0.487      | 19.695         | 0.0056      |
| Oxalacetate                    | HMDB00223 | C00036   | 0.557      | 13.771         | 0.0007      |
| Carnosine                      | HMDB00033 | C00386   | 0.582      | 21.161         | 0.0037      |
| N2, N2-dimethylguanosine       | HMDB04824 |               | 0.704      | 13.740         | 0.0056      |
| Dimethylglycine                | HMDB00092 | C01026   | 0.707      | 16.249         | 0.0013      |
| 2'-Deoxycytidine               | HMDB00014 | C00881   | 0.744      | 17.496         | 0.0011      |
| Uracil                         | HMDB00300 | C00106   | 0.773      | 20.861         | 0.0099      |

(Continued)
TB-peptide provides therapeutic benefits to alleviate sepsis-induced cardiomyopathy not only in young adults but also in aged population (Figure 1). Further, because autophagy intimately interacts with metabolic regulation,37,38 we examined whether lipopolysaccharide challenge and the following TB-peptide treatment alter cardiac metabolism in young and aged mice by a targeted approach of metabolomic analysis. Our data revealed that a toxic challenge of lipopolysaccharide triggers both age-dependent and age-independent reprogramming in energy metabolism in myocardium, and the effects of TB-peptide involve mitigating lipopolysaccharide-induced disturbance of carbohydrate and AA metabolism (Figures 2 through 4).

In sepsis, energy deficits, shown by abnormal accumulation of intermediates from breakdown of carbohydrates, lipids, and protein reserves, was found to associate with worsening outcomes, especially in non-survivors.39,40 Sepsis responses such as high fever, the activation of immune cells, tachycardia, tachypnea, and the acute production of reactants demand a higher level of energy supplies. Evidence supports the hypothesis that, during the phase of early sepsis, a hypermetabolic response enables the body’s defense mechanism to meet the needs of fighting against infection. However, late-stage sepsis is accompanied by hypometabolism leading to a severe disruption of metabolic homeostasis and creating a problem of metabolic deficiency. Prolonged hypometabolism is maladaptive because it generates a variety of toxic materials that stimulate inflammation and eventually provoke cell death and multiorgan dysfunction.10,11

Because autophagy is a self-survival mechanism via its “self-eating” capacity, promoting autophagy provides an opportunity to recycle the unwanted materials, such as damaged subcellular organelles, macro- and small molecules, that are used as replenished supplies for new biosynthesis.41 Indeed, strategies that boost autophagy have been shown to have therapeutic promise in animal disease models including sepsis.18,21,25,32 Testing potential therapeutic approaches in aged subjects is generally more challenging because of significantly reduced tolerance to stress conditions, likely a result of compromised immunity. Aged hearts are characterized as having decreased autophagy, accumulated mitochondrial damage, and higher vulnerability to acute insults such as sepsis.42–44 Consistent with the hypothetical benefit of autophagy, overexpression of autophagy genes or long-term application of pharmacological autophagy inducers increased life span in various animal models.33,45 In this present study, we obtained evidence showing that activation of autophagy by TB-peptide, when given post lipopolysaccharide-challenge, was able to improve cardiac performance and mitigate cytokine production in aged mice (Figure 1). The data suggest that a short-term application of autophagy inducer may effectively control sepsis-induced cardiomyopathy not only in young adults but also in an aged population.

One critical role of autophagy is to catalytically promote metabolic homeostasis under stress or disease conditions to meet the higher energy demand. In particular, autophagy is found to mediate the availability of carbohydrates, lipids, and nucleic acids through

### Table 6. Continued

| Metabolite                      | HMDB.ID       | KEGG.ID | logFC     | log2 abundance | FDR      |
|--------------------------------|---------------|---------|-----------|----------------|----------|
| UMP                            | HMDB0000288   | C00105  | 0.888752926 | 20.76603231    | 0.034403039 |
| 2-Hydroxyisobutyrate/2-Hydroxybutyrate | HMDB00729     |         | 0.925103751 | 18.70370022    | 0.013945565 |
| isoValeric acid/4-oxobutanoate/ acetoacetate | HMDB00718/ HMDB0001259 | C08262/ C00232/ C00164 | 0.929126079 | 15.83124364 | 0.098653456 |
| 3-Hydroxyisovaleric acid        | HMDB00754     | C02872  | 0.947558894 | 16.50883966    | 0.003901658 |
| 1-Methyladenosine               | HMDB003331    | C02494  | 1.047461411 | 16.60747522    | 0.00175991 |
| Pimelolate                      | HMDB00070     | C00408  | 1.093309517 | 18.29659999    | 0.007891768 |
| N-AcetylGlycine                 | HMDB00532     | C02878  | 1.357470336 | 15.9663203     | 0.005774246 |
| Suberic acid                    | HMDB00893     | C00475  | 1.39946568  | 21.73604314    | 1.08047E-06 |
| Cytidine                       | HMDB00089     | C01551  | 2.263705109 | 20.63393545    | 0.000125369 |
| Azelaic acid                    | HMDB00784     | C08261  | 1.596727785 | 18.01963077    | 0.007585928 |
| 3-Indoxyl sulfate               | HMDB00682     |         | 1.761797821 | 17.91025929    | 0.027607025 |
| N-Carbamoyl-B-alanine           | HMDB00026     | C02642  | 1.973807696 | 14.57996584    | 0.00957627 |
| 7-Methylguanine                 | HMDB00897     | C02242  | 2.106813609 | 15.09317898    | 0.000388829 |
| Pseudouridine                   | HMDB00767     | C02067  | 2.173259529 | 19.02450924    | 0.00028785 |
| Allantoin                       | HMDB00462     | C01551  | 2.263705109 | 20.63393545    | 0.000125369 |

FC indicates fold change; FDR, false discovery rate; and UMP, uridine 5'-monophosphate.
# Table 7. Lipopolysaccharide-Induced Significant Changes in Myocardial Metabolites of Old Mice Receiving TB-Peptide

| Metabolite                        | HMDB.ID         | KEgg.ID  | logFC     | log2 abundance | FDR        |
|----------------------------------|-----------------|----------|-----------|----------------|------------|
| Methionine Sulfoxide             | HMDB02005       | C02989   | −1.3153   | 0.032800624    |
| Guanosine                        | HMDB00133       | C00387   | −1.3184   | 0.002429568    |
| Arginine                         | HMDB00517       | C00062   | −1.2137   | 0.011751792    |
| 2-Aminoisobutyric acid           | HMDB01906       | C03665   | −1.1592   | 0.006785659    |
| Inosine                          | HMDB00196       | C00294   | −1.1140   | 0.001187937    |
| Aspartic acid                    | HMDB00191       | C00049   | −1.0665   | 0.002429568    |
| Adenine                          | HMDB00034       | C00147   | −1.0546   | 0.001187937    |
| gamma-Aminobutyrate              | HMDB000112      | C00334   | −1.0368   | 0.001187937    |
| Glucose                          | HMDB00122       | C00031   | −0.9260   | 0.030927335    |
| Lactose/trehalose                | HMDB00186       | C00243   | −0.8470   | 0.002429568    |
| Glutamic acid                    | HMDB000148      | C00025   | −0.8294   | 0.001187937    |
| Acetylphosphate                  | HMDB01494       | C00227   | −0.8216   | 0.002429568    |
| Serine                           | HMDB00187       | C00065   | −0.8135   | 0.013011786    |
| Ribose-5-P                       | HMDB01548       | C00117   | −0.7457   | 0.02328568     |
| Ribulose 5-phosphate             | HMDB00618       | C00199   | −0.7045   | 0.013011786    |
| Retinol                          | HMDB00305       | C19962   | −0.7396   | 0.011751792    |
| Phosphocreatine                  | HMDB01511       | C02305   | −0.7353   | 0.061264672    |
| Hypoxanthine                     | HMDB000157      | C00262   | −0.7203   | 0.002865168    |
| Cholesterol sulfate              | HMDB00653       | C18043   | −0.7110   | 0.077031359    |
| N6-Acetyl-lysine                 | HMDB00206       | C02727   | −0.7056   | 0.045328886    |
| Mannitol                         | HMDB00765       | C00392   | −0.6937   | 0.077782887    |
| Alanine                          | HMDB00161       | C00041   | −0.6827   | 0.005694127    |
| Adenylosuccinate                 | HMDB000538      | C03794   | −0.6796   | 0.079513095    |
| Creatine                         | HMDB000064      | C00300   | −0.6711   | 0.021813171    |
| Taurine                          | HMDB00251       | C00245   | −0.6697   | 0.002577699    |
| Asparagine                       | HMDB00168       | C00152   | −0.6544   | 0.030927335    |
| Taurocyamine                     | HMDB003584      | C01959   | −0.6497   | 0.033730157    |
| Linolenic acid                   | HMDB01388       | C06427   | −0.6426   | 0.050642956    |
| Pyroglutamic acid                | HMDB00267       | C01879   | −0.6387   | 0.04969271     |
| Methionine                       | HMDB00696       | C00073   | −0.5937   | 0.077031359    |
| isoLeucine/alloisoLeucine        | HMDB00172       | C00407   | −0.5777   | 0.021913171    |
| 3'-Methylthioadenosine           | HMDB00113       | C00170   | −0.5776   | 0.077031359    |
| Guanine                          | HMDB00132       | C00242   | −0.5513   | 0.030927335    |
| Nicarnamide                      | HMDB001406      | C00153   | −0.5480   | 0.030927335    |
| Hydroxyproline                   | HMDB00725       | C01157   | −0.5419   | 0.030927335    |
| CMP                              | HMDB00095       | C00055   | −0.5304   | 0.077031359    |
| N-acetyl-aspartate (NAA)          | HMDB00812       | C01042   | −0.5151   | 0.030927335    |
| Threonine                        | HMDB00167       | C00188   | −0.5133   | 0.077782887    |
| Choline                          | HMDB00097       | C00114   | −0.4977   | 0.004912092    |
| FAD                              | HMDB00124       | C00016   | −0.4852   | 0.050905372    |
| Betaine                          | HMDB00043       | C00719   | −0.4478   | 0.097856148    |
| Xanthine                         | HMDB00292       | C00385   | −0.4100   | 0.032800624    |
| N-Ac-alanine                     | HMDB00766       | C00881   | 0.4587    | 0.061217759    |
| 2'-Deoxyxycytidine               | HMDB00014       | C00861   | 0.4587    | 0.061217759    |
| 1-Methyladenosine                | HMDB00331       | C02494   | 0.5570    | 0.097733496    |
| Uracil                           | HMDB00300       | C00106   | 0.6286    | 0.002429568    |
| Glutaric acid                    | HMDB00661       | C00489   | 0.7407    | 0.054616457    |

(Continued)
selective signaling of glycophagy, lipophagy, DNAutophagy, and RNAutophagy, respectively. Therefore, reprogramming cardiac metabolism is an expected response to the challenge of lipopolysaccharide, as well as to the treatment of TB-peptide. In this report, an established targeted metabolic approach was applied to examine major metabolic pathways of energy production using substrates of carbohydrates, AAs, and lipids. Our data suggest that endotoxemia shock caused an age-independent downregulation in glucose metabolism and AA metabolism, shown by changes in glucose, UDP-glucose, L-methionine, aspartate, and glutamate (Figures 2 and 3, Tables 3 and 6). This detected effect of endotoxemia on carbohydrate metabolism is consistent with previous report of lipopolysaccharide-induced impairment in myocardial glucose metabolism in an ex vivo perfused heart model. We also found that TB-peptide exerted a reversing effect on these lipopolysaccharide-induced changes in metabolites, resulted in improved glucose and AAs metabolisms (Figures 2 and 3, Tables 4 and 7). It is worth pointing out that TB-peptide appears to have a stronger effect on AA metabolism, as summarized in the heatmap cluster analysis in Figure 4. In particular, TB-peptide protected metabolites generated via the glutamate–aspartate pathway from declines triggered by lipopolysaccharide.

Additionally, our data suggest that lipopolysaccharide challenge and the subsequent treatment of TB-peptide incite age-dependent responses of lipid metabolism in the heart. In the young group, lipopolysaccharide elevated levels of metabolites from lipid metabolism, such as glycolipids and linoleic acid (Figures 2 and Table 3). This observation is consistent

| Metabolite                        | HMDB.ID   | KEGG.ID | logFC       | log2 abundance | FDR        |
|-----------------------------------|-----------|---------|-------------|----------------|------------|
| 3-Hydroxyisovaleric acid          | HMDB00754 | C02867  | 0.786584264 | 16.50883966   | 0.030927335|
| Cytidine                          | HMDB00089 | C00475  | 0.799060305 | 21.73604314   | 0.00352042 |
| UMP                               | HMDB000288| C00105  | 0.965194771 | 20.76033231   | 0.033730157|
| Cystathionine                     | HMDB00099 | C02291  | 1.059338163 | 14.25041028   | 0.013011786|
| Azelaic acid                      | HMDB00784 | C08261  | 1.113138017 | 18.01963077   | 0.079513096|
| Glutaryl carnitine                | HMDB13130 | C00956  | 1.175174675 | 16.87943741   | 0.030927335|
| 2-Amino adipate                   | HMDB001138| C00624  | 1.403529606 | 14.71640701   | 0.030927335|
| N-Ac-glutamate                    | HMDB00682 | C01551  | 1.800469573 | 20.63393545   | 0.002865188|
| 3-Indoxyl sulfate                 | HMDB00462 | C02242  | 1.822720726 | 15.09317898   | 0.00352042 |
| Allantoin                         | HMDB00697 | C00624  | 1.924862053 | 19.02459212   | 0.002577699|
| 7-Methylguanine                   | HMDB00767 | C02067  | 1.984705127 | 14.57996584   | 0.02144052 |
| Pseudouridine                     | HMDB00026 | C02642  | 1.147225743 | 17.65586613   | 0.02110222 |
| N-carbamoyl-B-alanine             | HMDB00876 | C05443  | −1.374166031 | 14.2200854    | 0.063927243|
| Cholecalciferol                   | HMDB00205 | C02989  | −1.337128135 | 15.29682122   | 0.079081037|
| Methionine sulfoxide              | HMDB00653 | C18043  | −0.943757919 | 19.1375507    | 0.063927243|
| Cholesteryl sulfate               | HMDB01494 | C00227  | −0.66582046 | 16.39466205   | 0.0392488283|
| Acetylphosphate                   | HMDB00058 | C00575  | −0.656493835 | 14.58770357   | 0.085577179|
| cAMP                              | HMDB00064 | C00300  | −0.597420557 | 27.3962819    | 0.083469536|
| Creatine                          | HMDB00251 | C00245  | −0.578446884 | 25.54480293   | 0.0392488283|
| Taurine                           | HMDB00161 | C00041  | −0.547137941 | 22.92888413   | 0.063927243|
| Alanine                           | HMDB00017 | C00411  | −0.531313224 | 21.5717755    | 0.079081037|
| Hydroxyproline                    | HMDB00725 | C01157  | −0.52688538 | 23.26240474   | 0.063927243|
| Ribose-5-P                        | HMDB01548 | C00117  | −0.52688538 | 23.26240474   | 0.063927243|
| 2-Aminoadipate                    | HMDB00510 | C00956  | 1.263245307 | 16.87943741   | 0.063927243|

FC indicates fold change; and FDR, false discovery rate.
with published results in literature. For example, a clinic investigation detected a significant lipid accumulation in the myocardium of sepsis nonsurvivors. Follow-up studies in animal models further suggest that this sepsis-associated phenomenon is likely caused by blocked fatty acid oxidation due to impaired regulation via transcription factor peroxisome proliferator-activated receptor-α. In our study presented herein, we found that the lipopolysaccharide-induced increases in lipid metabolites were attenuated by the treatment with TB-peptide (Figure 2 and Table 4). A plausible mechanism of this peptide-mediated effect is that promoting autophagy improves the clearance of wasted molecules and thus reduces lipid accumulation. In addition, a boost in autophagy may improve the overall QC of the mitochondria pool in the heart and thus enhance the use of fatty acid substrates for production of energy. However, in the aged hearts, we observed that lipopolysaccharide mediated a moderate yet significant downregulation trend in fatty acids,
with little effect of TB-peptide (Figure 3 and Tables 6 through 8).

Whether the lipopolysaccharide-mediated decrease in lipids is pathological to the aged hearts remains to be further investigated. One point to consider is that myocardial lipid accumulation and impairment of fatty acid use increase with age. Thus, because of a relatively higher baseline levels of lipids, lipid changes in the aged hearts may not be as dramatic as those in the young groups in response to external stimuli such as lipopolysaccharide. Similarly, postchallenge administration of TB-peptide to temporally induce autophagy is unlikely to affect lipid levels in the heart. Nonetheless, knowledge regarding the age-associated difference in fatty acid metabolism in septic hearts is still limited. Whether the expression and enzymatic activities of fatty acid metabolic factors alter with age, and whether these changes are reprogrammed in response to septic challenge and autophagy stimulation, are critical to better understand the mechanism-of-action of TB-peptide. Furthermore, in sepsis, dysfunctional mitochondria and disrupted lipid metabolism were also observed in mitochondria-enriched organs other than the heart, such as in muscle and liver. Measurements of levels of metabolic substrates of glucose, lactate, and pyruvate in a porcine model of endotoxemia suggest that myocardium and skeletal muscle share a similar pattern of changes. Thus, increasing autophagy capacity by activating Beclin-1 via TB-peptide may have an effect to alleviate muscle atrophy and liver dysfunction. This potential effect and its possible association with aging require further evaluation.

CONCLUSIONS

Taken together, in this report, we provided evidence showing that Beclin-1 activating TB-peptide possesses therapeutic potential for sepsis-induced cardiomyopathy in both young and aged populations. A pilot metabolic study using a targeted metabolomics analysis has linked this beneficial effect to improvements in carbohydrate and AA metabolism. Future studies are warranted to determine the functional changes of regulatory signaling factors in these events. Furthermore, given the limited number of metabolites measured in targeted metabolomic profiles (albeit with high sensitivity), application of an untargeted metabolomics approach may reveal a broader range of cardiac metabolites impactaffecteded by age, lipopolysaccharide, and TB-peptide. It is also recognized that sepsis-induced changes in metabolic homeostasis progresses with severity and depends on the context of tissue and/or cell types. For example, in a mouse model of endotoxemia, lipopolysaccharide challenge decreases lipid levels in the blood while increasing then in the liver, suggesting a possibility of transporting lipids to the liver as a potential energy source. Further, different types of shock conditions appear to stimulate distinct metabolic pathways; such difference was found in the heart and muscle when models of endotoxemia and hemorrhage shock were compared. Lastly, though the endotoxemia model has been widely used as an experimental model mimicking the overwhelming inflammation state at the initial phase of human sepsis, studies in models of infection-induced sepsis, such as cecal ligation and puncture sepsis or pneumonia sepsis, are expected to reveal more in-depth knowledge of relevance with clinical status and/or pathogen specificity. In a recent study, we obtained promising results suggesting that TB-peptide has a therapeutic potential in control of pulmonary pathology in a mouse model of pneumonia sepsis. Future evaluation of metabolic reprogramming at different sepsis models, stages of sepsis, and in different organs could help identify metabolic chemicals and/or regulatory enzymes as diagnostic markers and drug targets for sepsis. Eventually, studies in this area are expected to develop strategies for improving metabolic plasticity as potential new and effective therapies.

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Received January 7, 2022; accepted June 2, 2022.

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Sources of Funding

This work is supported by Nathan Shock Center Pilot Award (to Zang), National Institutes of Health (NIH) grant 2R01GM111295-01 (to Zang), HL109471 and CA215063 (to Liu), American Heart Association grant AHA 19TP34910172 (to Liu), NIH R01HL158515 and R01GM124108 (to Li), R01AG049494 (to Promislow), NIH P30 AG012880 (to the University of Washington Nathan Shock Center), and NIH S10 Grant 1S10OD021562-01 (to Raftery) which funded a purchase of the LC–MS system used to acquire targeted metabolomics data. The NIH Common Fund’s National Metabolomics Data Repository website, the Metabolomics Workbench, is supported by NIH grant U2C-DK119886.

Disclosures

None.

Supplemental Material

Data S1

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Data S1. Evaluation of data quality, exploratory analysis, and data preprocessing

To remove the systematic variation between samples, we performed a median normalization such that all the samples have the same median value post log2 transformation. Below are box plots of before and after normalization.
1) Date Filtering

This project used a targeted Mass Spec analysis designed to detect 361 metabolites. 199 of these metabolites were not detected in any of the samples and, therefore, had 100% missingness. The overall missingness of the remaining 162 metabolites was 1.2%. The figure below shows the percent missingness versus the mean log2-abundance for each metabolite.

We selected 156 metabolites with missingness < 20% and a coefficient of variation (CV) < 20% in the pooled sample QC data. After filtering, no missing values remained, therefore, no imputation was performed.
2) Evaluation of data quality-principal components analysis (PCA)

PCA is a method to take high dimensional data and reduce it to only a few dimensions to visualize how similar samples of the same type are. A series of PCA plots was generated using the log2 transformed, normalized, and filtered data. Potential variations driven by protein amount or run order were not observed detected.