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Salutary effects of Resveratrol on sepsis-induced myocardial depression

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Abstract

Objectives: We hypothesized that resveratrol (ResV) administration would reverse sepsis-dependent down-regulation of peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α), preserve mitochondrial integrity and rescue animals from sepsis-induced myocardial failure.

Setting: Teaching hospital research laboratory.

Interventions: Cecal ligation and puncture (CLP) in mice was performed to induce sepsis. Mice that underwent CLP were randomly assigned to receive resveratrol (30 or 60 mg/kg) or vehicle 1 ml NaCl 0.9% subcutaneously in the scruff of the neck directly after surgery and at 16, 24 and 40 hours (h).

Measurements and Results: Forty-eight hours after CLP, cardiac performance was established using echocardiography. Mitochondrial integrity was evaluated with electron microscopy and changes in gene expression were evaluated with microarray analysis. Survival at 48 h was just under 50% and comparable between groups. Myocardial contractile function significantly improved after ResV treatment. ResV treated mice developed focal areas of edema, whereas vehicle treated mice developed significant diffuse myocardial edema. EM revealed widespread swollen mitochondria with ruptured outer membranes, autophagosomes and vacuolation of the internal compartment, which were significantly attenuated in ResV-treated animals. ResV treatment significantly increased cardiac expression of PGC-1α. Microarray analysis revealed that ResV treatment resulted in up regulation of the PGC gene set containing genes known to be regulated by PGC-1α. Our data strongly suggest that administration of ResV modulates bioenergy metabolism, substrate utilization, oxidative stress and detoxification pathways associated with both mitochondrial and cardiac pathological conditions.

Conclusions: The salutary effects of ResV on CLP-induced myocardial dysfunction are associated with increased PGC-1α abundance and function. Preservation of myocardial energy production capacity, prevention of secondary injury, mitigation of inflammation and reversal of sepsis-induced myocardial remodeling are likely to underlie its beneficial effects.
**Introduction**

The mechanisms underlying myocardial depression in sepsis are incompletely understood. However, since full recovery of cardiac function is seen in survivors at 7–10 days (1), it seems that functional rather than structural changes are responsible for intrinsic myocardial depression (2). At a subcellular level, as sepsis-induced energy demands outstrip the supply, adaptive changes are presumed to trigger a molecular shift in the cellular substrate utilization and contractile function. Down-regulation of bioenergy requirements is hypothesized to enable cardiomyocytes to circumvent death thus explaining improved survival in prolonged sepsis. In this context, sepsis-induced mitochondria dysfunction and damage appears to be the critical modulating event coordinating cellular adaptive responses (rather than maladaptive) to compounding stressors (3). Indeed, the degree of mitochondrial dysfunction measured in skeletal muscle biopsies correlates with outcome in patients with severe sepsis (4-6). Furthermore, mitochondrial damage in the heart has been found in human autopsy specimens of patients with severe sepsis (7).

Perixisome proliferator-activated receptor (PPAR)-γ coactivator (PGC)-1α is a transcriptional coactivator that plays a key regulator role in mitochondrial metabolism and biogenesis. It functions as a transcriptional co-activator for various transcription factors (TF) which are involved in energy metabolism and muscle fiber expression (8,9). We have recently shown that the molecular switch favouring the expression of fetal isoforms of contraction related proteins known to occur after sepsis-induced myocardial depression is associated with down regulation of PGC-1α and related TFs in an iNOS dependent manner. In several cardiac pathological states, down regulation of PGC-1α and its target genes is shown to correlate with severity (10-12).

Resveratrol (ResV) is a polyphenol, mostly known as a constituent of red wine, that has positive effects in a wide variety of disease states (13,14). It has been implicated in the regulation of expression and activation of key TFs and their co-factors such as PGC-1α (15,16). In skeletal muscle, ResV treatment results in up-regulation of PGC-1α and consequent increase in mitochondrial size, DNA content and enhanced mitochondrial enzymatic activity.

In this study we hypothesized that RSV administration would reverse sepsis-dependent down-regulation of PGC-1α, preserve mitochondrial integrity and rescue animals from sepsis-induced myocardial failure. We performed cecal ligation and puncture (CLP) in mice to induce chronic sub-lethal polymicrobial sepsis. Forty-eight h after CLP, cardiac performance was established using echocardiography. Mitochondrial integrity was evaluated with electron microscopy (EM) and changes
in gene expression associated with RSV administration and improvement of myocardial function was evaluated with microarray analysis and quantitative real-time polymerase chain reaction (qRT-PCR).

Materials and Methods

Detailed methodology is presented in the supplements.

Animal Experiment: Male C57/BL6 mice (8-10 weeks, Wild type; Jackson Laboratory, Bar Harbor, ME, USA) were randomized to caecal ligation and perforation (CLP) or sham operation. Experiments were conducted in accordance with standard operating procedures of the Department of Comparative Medicine University of Toronto, Toronto, Canada. Protocol was approved by Institutional Animal Care and Use Committee at St. Michael’s Hospital. Mice were randomized to Resveratrol (30 or 60 mg/kg) (Cat#554325, Calbiochem, Darmstadt, Germany) or vehicle 1 ml saline subcutaneously after surgery and at 16, 24 and 40 h. Dose and timing was determined by preliminary experiments. At 48h mice underwent echocardiography, and were sacrificed by cardiac puncture. Hearts was removed fixed or snap-frozen. Investigators performing assessments were blinded to treatment assignment.

Cardiac Echocardiography: Heart rate, left ventricular end diastolic diameter (LVEDD), end systolic diameter (LVESD), end diastolic area (LVEDA) and end systolic area (LVESA) were measured. Fractional shortening (FS), fractional area change (FAC) and left ventricular ejection fraction (LVEF) were calculated.

Pathology: Scoring of tissue injury was performed on formalin fixed paraffin embedded whole heart sections (4 mice randomly selected from each group) stained with hematoxylin and eosin (HE) (17). Ultrastructural changes were determined by an electron microscopy (EM) muscle pathologist blinded to group assignment (N=3 animals/group; 5-8 sections/animal). Qualitative and semiquantitative injury assessment was adapted from Bishop et al. (18). The grading data were not subjected to statistical analysis.

RNA isolation and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR): Total RNA from whole hearts was isolated and purified (17). Primer sequences are in Supplemental Table 1. Expression was normalised to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and/or beta-actin (β-actin).

Microarray Analysis: A total of 300 ng of mRNA from whole hearts (4 animals/group) was hybridized to Illumina Mouse WG 6v1.1 chip. Data was processed using R-Project for Bioconductor (19) and normalised using variance-stabilizing transformation (VST) (20). We performed one-class analysis in SAM (significant analysis of microarray) (21) to filter out non-specific genes. A total of 18,586 probes
significantly changed relative to the standard deviation of repeated measurements of each probe across all experiments (false discovery rate (FDR) < 0.9%, delta 7.1888) were used for gene set enrichment analysis (GSEA, http://www.broadinstitute.org/gsea/index.jsp) (22-24). A FDR of 25% was used as the cut-off for significance (23,25). Specific pathways were visualized using Ingenuity Pathway Analysis (IPA, Ingenuity Systems, Inc. Redwood City, CA).

Primary cardiomyocyte isolation and protein detection: Primary cultures of neonatal cardiomyocytes were prepared from Sprague-Dawley rats (26,27). Once isolated, cardiomyocytes were treated with lipopolysaccharide (LPS, 1mg/ml), ResV (50 mM), LPS+ResV or vehicle alone for 48 h. The relative abundance of PGC-1α, nuclear factor erythroid-derived 2-like (Nrf)-2 (C-20), and b-actin was detected by Western blot (17,28).

Antibodies: PGC-1α, Nrf-2 (C-20), b-actin and horseradish peroxidase-conjugated secondary antibodies to mouse or rabbit immunoglobulins were purchased from Santa Cruz Biotech (Santa Cruz, CA). Other chemicals were purchased from Sigma or Fisher Scientific.

Statistics: No differences in mortality were found between the two dosages of ResV using log rank test; data were therefore analyzed collectively (supplemental Figure 1). Independent samples t-test were performed for qRT-PCR data. Data are expressed as mean ± standard error of the mean (SEM). A P-value <0.05 was considered significant.

Results

Effects of ResV on Survival. Animals became lethargic, stopped grooming and developed diarrhea after CLP. No behavioral difference was seen between vehicle and ResV treated mice. Forty-eight hours after the induction of sepsis, weight loss was not different between the groups (data not shown). Survival in the vehicle-treated group was 48 % and treatment with ResV (30 or 60 mg/kg) did not lead to a significant improvement (Figure 1A and supplemental Figure 1A). Assessment of myocardial function was obtained for thirty-three surviving mice (17 CLP + vehicle and 16 CLP + ResV, supplemental Figure 1B).

ResV administration improves myocardial contractility. At 48 h, exposure to CLP resulted in decreased myocardial contractility in the vehicle compared to the ResV treated group. ResV treated mice had better fractional shortening (P = 0.007) (Figure 1B), fractional area change (P = 0.017) (Figure 1C), and ejection fraction (P = 0.011) (Figure 1D) than vehicle treated animals (Supplemental Figure 1B). No differences were found in LVEDD and LVEDA. Heart rate did not differ between the groups (Figure 1E).
**Left page; Figure 1. Effect of ResV treatment on survival and myocardial function and structure.** (CLP = cecal ligation and puncture, ResV = resveratrol, LV = left ventricle) A. Kaplan-Meir survival curves for mice exposed to CLP treated with vehicle or ResV. Survival in the vehicle-treated group at 48 h was just under 50%. Treatment with ResV did not lead to a significant improvement in survival. Data represents percentage survival of vehicle- (CLP + vehicle = 17) and ResV (CLP + ResV = 16) -treated mice 48 h after induction of polymicrobial sepsis by CLP. B. Fractional shortening (P = 0.007); C. Fractional area change (P = 0.027); D. Ejection fraction (P = 0.013) of the left ventricle; and E. Heart Rate (HR; P=0.08). Data is presented as the average values for each group. Error bars represent the standard error of the mean (SEM).

**Resveratrol in the septic heart**

**Left page; Figure 1. Effect of ResV treatment on survival and myocardial function and structure.** (CLP = cecal ligation and puncture, ResV = resveratrol, LV = left ventricle) A. Kaplan-Meir survival curves for mice exposed to CLP treated with vehicle or ResV. Survival in the vehicle-treated group at 48 h was just under 50%. Treatment with ResV did not lead to a significant improvement in survival. Data represents percentage survival of vehicle- (CLP + vehicle = 17) and ResV (CLP + ResV = 16) -treated mice 48 h after induction of polymicrobial sepsis by CLP. B. Fractional shortening (P = 0.007); C. Fractional area change (P = 0.027); D. Ejection fraction (P = 0.013) of the left ventricle; and E. Heart Rate (HR; P=0.08). Data is presented as the average values for each group. Error bars represent the standard error of the mean (SEM).

**Treatment with ResV attenuates CLP-induced myocardial ultrastructural injury.** After CLP-induction, hearts from vehicle treated mice showed grade 2-3 injury, i.e. focal lesions and myocardial edema, extending over a wider area of the left ventricle with right ventricular involvement (Supplemental Figure 2). ResV treated mice developed grade 1-2 injury (focal areas of edema), but were generally spared the diffuse myocardial edema. EM revealed ultrastructural changes in mitochondria from mice that developed moderate to severe myocardial depression (17) (Figure 1F). Representative images from vehicle-treated mice demonstrated widespread swollen mitochondria with ruptured outer membranes (arrowheads). Matrix paling and disrupted cristae was associated with vacuolation of the internal compartment. These changes were significantly attenuated in ResV-treated animals (Table 1).

**Transcriptional Response to ResV in CLP-induced myocardial depression.** We used GSEA to look for coordinated expression within treated samples of 18,586 most variable probes filtered using one-class analysis in SAM (corresponding to 14,828 genes, FDR < 0.9%, delta 7.1888). ResV administration resulted in up-regulation of 121 gene sets, including various involved in bioenergy metabolism such as oxidative phosphorylation, mitochondrial, ribosomal structure and function, and pyrimidine metabolism (Figure 2, Table 2 and Supplemental Table 2). ResV treatment resulted in up-regulation of gene sets associated with known pathological mitochondrial neurological dysfunction conditions (Parkinson’s, Alzheimer’s and Huntington’s disease), detoxification and management of oxidative stress. The putative impact of changes in gene expression in specific pathways was visualized using

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Chapter 4

IPA (Figure 3). Treatment with ResV leads to specific up-regulation of individual components of Complex I, II and III, as well as partial up-regulation of complex IV and Y secretases (combined gene list presented in Supplemental Table 4 and 5).

In contrast, down-regulated gene sets (54 gene sets) included sets previously known to play a role in pathological heart conditions associated with arrhythmias, sodium reabsorption, dilated and hypertrophic cardiomyopathy (Figure 4, Table 3, supplemental Tables 3 and 6). ResV treatment also led to down regulation of critical pathways involved in insulin signaling (Supplemental Figure 3 and Table 7), mitogen-activated protein kinase signaling (Supplemental Figure 4 and Table 8), myocardial inflammation (viral myocarditis) and response to stress (Table 3 and Supplemental Table 3).

ResV treatment resulted in enrichment for genes known to be regulated by PGC-1α (Figure 5). We compared these genes with genes known to be PGC-1α responsive in the MitoCarta inventory of mammalian mitochondrial genes (29). Data for all genes enriched in sepsis-induced myocardial

<table>
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<tr>
<td>Mitochondria</td>
<td>The structural integrity of mitochondria is compromised. Swelling, rarefaction, vacuolation, and appearance of electron dense granules (EDG). Occasional lipid droplets, homogenization of cristae, membrane calcification and empty spaces left following destruction of mitochondria and myofibrils. Mitochondrial injury affects more than 50-75% of mitochondria.</td>
<td>The structural integrity of the mitochondria is well preserved apart from some infrequent homogenization of cristae and signs of early calcification of mitochondrial membranes. Mild to moderate swelling, vacuolization, less commonly total break-up of the organelles, resulting in formation of optically empty spaces. Mitochondrial injury occurs in an estimated 25-35% of the organelles.</td>
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<td>Myofibrils</td>
<td>Increased electron density of myofibrils (especially of the Z-disc) in areas of injury. Focal myocardial necrosis. Multiple foci of separation of myofibrils from the intercalated disc ruptured from myocardium.</td>
<td>Multiple foci of separation of myofibrils from the intercalated disc.</td>
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Table 1. Ultrastructural Assessment of Myocardial Morphology/Injury in Murine Hearts. Table describing electron microscopy assessment of morphological and ultrastructural changes in murine hearts exposed to a model of polymicrobial sepsis (CLP) treated with either vehicle or Resveratrol (ResV). The alterations observed in the individual animals are rated as 1 (normal) to 4 (severe).
depression after ResV treatment and MitoCarta documented PGC-1α induction is presented in Supplemental Table 9. The top network identified by IPA as ResV responsive identified PGC-1α dependent genes specifically involved in neurological diseases, skeletal and muscle disorders (Figure 5C).

Our data strongly suggest that administration of ResV restores transcription of bioenergy metabolism, substrate utilization, oxidative stress and detoxification pathways associated with both mitochondrial and cardiac pathological conditions.

**Effects of ResV on the expression of PGC-1α related genes and TFs.** We assessed the expression of PGC-1α related genes and TFs by qRT-PCR in whole hearts collected at 48 h (Figure 6). Relative change in gene expression is expressed as a ratio of gene expression in treated (CLP) versus sham samples (non-septic mice). Expression values were normalised to glyceraldehyde 3-phosphate dehydrogenase.
Table 2. Top canonical pathway related gene sets enriched in CLP + ResV (Upregulated by ResV). List of top canonical pathways gene sets (CP) from Molecular Signatures Database (MSigDB) found to be enriched in hearts from mice exposed to CLP treated with ResV (contains genes that are up-regulated in the presence of ResV). The CP MSigDB contains genes sets from the pathway databases including BioCarta, KEGG and biological process compiled by domain experts. GSEA Gene Set Name (CP); SIZE (size of gene set); enrichment score (ES), normalised ES (NES), nominal p value of the enrichment score for a single gene set (NOM p-val), and the false discovery rate (FDR q-val).

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Right page; Figure 3. Up-regulation of Genes involved in mitochondrial dysfunction. Genes contributing to mitochondrial related functional gene set enrichment were inputted into IPA. IPA library of canonical pathways identified mitochondrial dysfunction as a top pathway altered in murine septic hearts after treatment with ResV. Molecule from the data set (gene list presented in Supplemental Table 4) that were associated with Ingenuity’s Knowledge Base were considered for the analysis. The significance of the association and between the data set and the canonical pathway was determined in two ways: i) ratio of the number of molecules from the data set that map to the pathway divided by the total number of molecules that map to the canonical pathway (ratio 0.126) and ii) Fisher’s exact test was used to calculate a p-value determining the probability that the association between the genes in the data set and the canonical pathway can be explained by chance alone ($3.47 \times 10^{-16}$). By convention up-regulated genes are shown in red and down-regulated genes are shown in green. Legend for IPA diagram is presented with supplemental material.
Resveratrol in the septic heart
Resveratrol in the septic heart

Left page; Figure 4. Down regulation of genes involved in myocardial dysfunction A. GSEA enrichment plot for genes involved in dilated cardiomyopathy (GSEA KEGG pathways, Table 3). As described in Figure 2, top part of each plot shows the progression of the running enrichment score (ES) and the maximum peak therein. The middle part shows the genes in the gene set as “hits” against the ranked list of genes. The lower part shows the histogram for the ranked list of all genes in the expression data set. B. The corresponding heat maps show the expression values for the top subset of genes in each pathway that contributes to the enrichment score in the 8 mice profiled. Results are converted to colour, where red indicates a high and blue a low expression value. False discovery rate (FDR); Gene symbols are described in Supplemental Table 6. C. IPA library of canonical pathways identified myocardial hypertrophy as a top pathway altered in murine septic hearts after treatment with ResV (ratio 0.069, p-value 8.0E-17). Figure legend as described in Figure 3.

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Chapter 4

**A**

FDR q-value = 0.1046

**B**

- **Growth and muscle fiber expression**
- **Mitochondrial replication**
- **Electron transport**
- **OXPHOS**
- **Glucose oxidation**
- **Fatty acid oxidation**
- **Oxidative stress**
- **Cell cycle/death**
- **Muscle fiber expression**

**C**

Neurological disease, skeletal and muscular disorder

**D**

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Resveratrol in the septic heart

Left page; Figure 5. Enrichment for PGC-1α related genes. A. GSEA enrichment score plot for PGC-1α related genes (legend described in Figure 2), B. Schematic overview of top PGC-1α related transcription factors and putative roles in bioenergy metabolism and contractile function. C. Top network enriched for PGC-1α related genes. Genes that contributed to enrichment of the PGC-1α gene set and that were also known to be modulated by PGC-1α as per the MitoCarta inventory of mammalian mitochondrial genes (gene list and induction by PGC-1α as documented during the MitoCarta experiments is presented in Supplemental Table 9) were inputted into IPA. A network is a graphical representation of the molecular relationships between molecules. Molecules are represented as nodes and the relationship between two nodes is represented as an edge (line). Top network identified as PGC-1α responsive identified molecules known to be involved in both neurological diseases and skeletal or muscle disorders (legend for IPA schematic is available with supplemental material). D. The corresponding heat maps show the expression values for the top subset of genes in each pathway that contributes to the enrichment score in the 8 mice profiled. By convention red is up regulated and green is down regulated (genes from the heat map presented in the network are highlighted in bold).

(Gapdh). ResV treatment significantly increased the expression of PGC-1α, iNOS and Nos3 and Forkhead box O (Foxo)-3. Expression of myocyte enhancing factor (Mef)-2c and nuclear factor erythroid-derived 2-like 2 (Nrf2) was significantly lower in the ResV treated animals.

Gene Set Enrichment Analysis of cis-regulatory motifs. To identify common features amongst ResV regulated genes we used GSEA to screen the 4-kb segment centered on the transcription start site and 3’ region for known TF binding sites contained in the Molecular Signatures Database (MolSigDB, C3 database). While a total of 174 gene sets for known putative TF binding sites were enriched in the vehicle treated group (Supplemental Table 10), only 3 gene sets were enriched by treatment with ResV, all for unknown putative TF binding sites. Down regulated gene sets were enriched for PGC-1α related TFs: Nrf-2, Mef-2, Foxo-3, Foxo-4, Err-1 and PPARγ. Importantly, treatment with ResV also led to down regulation of putative inflammation related TFs nuclear factor-κ beta (NF-κB), activator protein (AP)-1 and CCAAT-enhancer-binding proteins (CEBP) binding sequences (Supplemental Table 10 and supplemental Figure 5). Given the significant overlap in TF binding sites, our data strongly suggests that in our model, the salutary effects of ResV on myocardial depression may be PGC-1α and inflammation dependent.

PGC-1α and Nrf-2 protein expression in heart tissues and cardiomyocytes. Exposure to ResV results in increased PGC-1α and decreased Nrf-2 expression which was confirmed by Western blot in heart tissue from wild type mice exposed to CLP treated with vehicle or ResV for 48h (Figure 6B). To determine the effects of ResV treatment on primary cardiomyocytes, cells were isolated from neonatal Sprague-Dawley rat hearts. Primary cardiomyocytes were treated with lipopolysaccharide (LPS, 1mg/ml), Resveratrol (ResV, 50 mM), LPS+ResV or vehicle alone for 48 h. Untreated (control) cells express basal levels of PGC-1α and Nrf2. Similar to results from tissues, ResV treatment results in increased PGC-1α and decreased Nrf2 expression. In cells exposed to LPS, PGC-1α expression is reduced and Nrf-2
Figure 6. Expression of PGC-1α and related proteins: A. Quantitative real time PCR (qRT-PCR) determined changes in the expression of PGC-1α related genes in the hearts of CLP-vehicle compared to CLP-ResV treated animals. Relative changes in gene expression were normalised to GAPDH (glyceraldehyde 3-phosphate dehydrogenase) and the baseline sample CLP Sham. β-actin (B-actin), estrogen-related receptor 1 (ERR-1), Forkhead box O3 and 4 (FOXO-3 and 4), inducible nitric oxide synthase (iNOS), myocyte enhancer factor-2a and c (Mef2a and c), nitric oxide synthase 3 (NOS3 or Endothelial NOS (eNOS)), Nuclear factor erythroid-derived 2-like 2 (Nrf-2), Peroxisome proliferator-activated receptor gamma coactivator - 1α (PGC-1α), peroxisome proliferator-activated receptor α and γ (PPARα and PPARγ) and serum response factor (Srf). * Significant fold change is defined as P<0.05, T-test CLP-vehicle vs. CLP-ResV treated. Arrow bars are standard deviations.

B. Heart tissue from mice exposed to CLP treated with vehicle or ResV for 48h

C. Primary cardiomyocytes treated with vehicle, LPS, ResV and LPS+ResV for 48h

Figure 6. Expression of PGC-1α and related proteins: A. Quantitative real time PCR (qRT-PCR) determined changes in the expression of PGC-1α related genes in the hearts of CLP-vehicle compared to CLP-ResV treated animals. Relative changes in gene expression were normalised to GAPDH (glyceraldehyde 3-phosphate dehydrogenase) and the baseline sample CLP Sham. β-actin (B-actin), estrogen-related receptor 1 (ERR-1), Forkhead box O3 and 4 (FOXO-3 and 4), inducible nitric oxide synthase (iNOS), myocyte enhancer factor-2a and c (Mef2a and c), nitric oxide synthase 3 (NOS3 or Endothelial NOS (eNOS)), Nuclear factor erythroid-derived 2-like 2 (Nrf-2), Peroxisome proliferator-activated receptor gamma coactivator - 1α (PGC-1α), peroxisome proliferator-activated receptor α and γ (PPARα and PPARγ) and serum response factor (Srf). * Significant fold change is defined as P<0.05, T-test CLP-vehicle vs. CLP-ResV treated. Arrow bars are standard deviations.

B. Heart tissue from mice exposed to CLP treated with vehicle or ResV for 48h

C. Primary cardiomyocytes treated with vehicle, LPS, ResV and LPS+ResV for 48h
protein expression is increased. Treatment with ResV results in increased PGC-1α and decreased Nrf-2 protein expression (Figure 6C).

Discussion

In keeping with previous findings from our group, at 48 h, CLP induced significant decrease in myocardial contractile function (17). This was accompanied by moderate to severe loss of mitochondrial integrity, swelling, destruction of myofibrils associated with myocardial injury and decreased PGC-1α transcription, expression and function. Treatment with ResV increased PGC-1α expression and function, rescued mice from severe ultrastructural damage and preserved myocardial contractile function; however, it did not result in a change in mortality at 48 h.

Over the past decade, the issue of adaptive versus maladaptive responses to sepsis has led to the central thesis that decrease in mortality and recovery would be contingent upon adequate early maintenance, or subsequent restoration, of mitochondrial function to meet metabolic energy demands enabling cells to fulfil critical roles including calcium homeostasis, maintenance of cellular redox state, and cell signalling (30). Although we do not know the exact cause of death in the animals that died post CLP, our data suggests that in the first 2 days of severe sepsis, other factors (such as neuroimmunoregulatory responses), extending beyond preservation of contraction-dependent bioenergy metabolism may contribute to mortality. These findings may have important clinical and therapeutic implications, i.e. the molecular determinants of sepsis-induced cardiomyopathy may not be the same as the molecular determinants of death from sepsis-induced multiorgan failure.

We have previously shown that loss of PGC-1α mRNA and protein is a critical event associated with the advent and severity of sepsis-induced myocardial depression (17). Moreover, increase in PGC-1α abundance and function precedes recovery of mitochondrial function, metabolic rate, and physiologic and biochemical organ function (31). Exercise and physiological hypertrophy activate muscle PGC-1α by protein phosphorylation (32). This modification increases PGC-1α protein stability, promotes its nuclear translocation, and precedes increases in its mRNA abundance (33-35). In contrast, binding of PGC-1α to the p65 subunit of NF-kB (Rel a) represses PGC-1α activity leading to reduction in pyruvate dehydrogenase 4 (Pdk4) expression and subsequent increase in glucose oxidation observed during proinflamatory states (36). Although we did not document a change in the expression of Pdk4, ResV resulted in regulation of insulin dependent genes, and a decrease in Rel a expression, consistent with the increase in PGC-1α transcriptional activity we see at 48h.
In our model, treatment with ResV led to an increase in PGC-1α mRNA, protein expression and transcriptional activity. The effect of ResV on mitochondrial gene expression and related functions, including decrease in Nrf2 expression and activity (overall decrease in oxidative stress) likely reflects the beneficial effects of the drug on preservation of mitochondrial structural integrity, as evidenced by ultrastructural assessment in survivors at 48 h. It is unclear if the improvement in myocardial contractility and mitochondrial function is due to preservation of existing mitochondria or increased mitochondrial biogenesis and turnover. We documented increased expression of TFAM (transcription factor A mitochondria) and genes containing putative nuclear respiratory factor (NRF)-1 (mitochondrial biogenesis) binding regulatory sequences. But other mitochondrial related proteins critical to mitochondrial biogenesis such as PPARα and γ were not altered. Consistent with our findings, transcriptional activity favouring up-regulation of critical components of the respiratory complex subunits was significantly increased after ResV treatment and is associated with improved myocardial function and survival (16,37).

Although we saw dramatic changes in myocardial structure and function, it is still possible that we were not powered to detect a significant reduction in mortality after only 48 h. Alternatively, it is possible that myocardial depression is not the determining factor with respect to mortality from severe sepsis. In this study, we did not treat our animals with antibiotics. A more clinically relevant model may limit early deaths and unmask a significant effect of ResV on mortality at later time points. Further insight into this issue may be of fundamental importance to drug development for sepsis.

Using microarray analysis we were able to determine that ResV treatment favored up regulation of genes within pathways related to bioenergy metabolism important for reversing oxidative phosphorylation dysfunction including genes encoding for components of the electron transport chain. In contrast, down-regulated functional groups implicated genes involved in PGC-1α cotranscriptional activity such as Nrf-2, Mef-2, Foxo-4, Err-1 and PPAR. This is in agreement with the findings from GSEA that identified up-regulated gene sets involved with detoxification and down regulation of genes involved in pathological cardiac conditions. Our findings are widely supported by the literature implicating PGC-1α as a critical protection factor mitigating pathological cardiomyopathy and hypertrophy (32,38,39).

Of specific interest is the role of ResV in down regulating the proinflammatory response. In this study, genes involved in MAPK as well as NF-kB, AP1, nuclear factor of activated T-cells (NFAT) and CREB signaling were down regulated by ResV treatment. Various studies support an anti-inflammatory
role for ResV (40-42). This may in fact be related to ResV induced increased in nitric oxide synthesis (43). In the heart, ResV has been shown to increase NO production by enhancing endothelial NO synthase (eNOS) expression while simultaneously reducing nitrosilation by inhibiting key enzymes involved in this process (44). In our study, ResV also led to an increase in iNOS expression. Reports on the effects of ResV on iNOS activity are controversial. While some studies report ResV-induced decrease in iNOS expression (45), others report an increase in iNOS expression(46). In fact, ResV was unable to protect kidney, brain, and heart cells from ischemia-reperfusion injury in iNOS knockout mice (47).

Treatment with ResV has been shown to attenuate echocardiographic changes associated with pathological myocardial hypertrophy as well as dilatation (48-50). Salutary effects have been associated with its effect on cardiac remodeling (51,52). In keeping with our findings, ResV may also attenuate arrhythmias associated with myocardial injury (53).

In this study mitochondrial function was not measured. However, it has been shown that ResV increases citrate activity (54) and, as in our study, restores activity of mitochondrial complex I, II and III (55). Given the preservation of mitochondrial structure, increased transcriptional activity of genes involved in detoxification, management of oxidative stress, and oxidative phosphorylation as well as decrease in markers of increased oxidative stress (Nrf-2 activity), we would expect that the ResV-treated group will have a higher energy production than their disrupted counterparts in the vehicle-treated group. Moreover, given we were unable to examine the structure and function of hearts in those animals that died, our study is biased towards survivors. A time course experiment would enable critical understanding of molecular changes associated with increased mortality.

In conclusion, the salutary effects of ResV on CLP-induced myocardial dysfunction are associated with increased PGC-1α abundance and function. Preservation of myocardial energy production capacity, prevention of secondary injury, mitigation of inflammation and reversal of sepsis-induced myocardial remodeling are likely to underlie its beneficial physiological and histopathological effects. Future studies will be fundamental to determine if these effects can change outcome.
References


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Detailed Material and Methods

Animal Experiment. Male C57/BL6 mice aged 8-10 weeks (Wild type; Jackson Laboratory, Bar Harbor, ME, USA) were randomly assigned to cecal ligation and perforation (CLP) or sham operation as previously described (1). Experiments were conducted in accordance with standard operating procedures of the Department of Comparative Medicine University of Toronto, Toronto, Canada. Experimental protocol was approved by Institutional Animal Care and Use Committee at Saint Michael’s Hospital. Our objective was to establish a model of hemodynamically stable sepsis. Briefly, mice were anaesthetised with 100 mg/kg ketamine and 10 mg/kg xylazine administered intraperitoneally and weighed. Peritoneal cavity was opened, cecum was identified, ligated without limiting flow and punctured using a 25g needle. The cecum was then returned to the abdomen and abdomen was closed. Sham-operated mice underwent an identical procedure except that the cecum was mobilized but not ligated and perforated. All animals received fluid resuscitation (0.5 ml of saline) and pain management with buprenorphine (0.03 mg/kg) once daily. Mice that underwent CLP were randomly assigned to receive Resveratrol (60 mg/kg) (Cat#554325, Calbiochem, Darmstadt, Germany) or vehicle 1 ml NaCl 0.9% subcutaneously in the scruff of the neck directly after surgery and at 16, 24 and 40 hours. After 48h animals were anaesthetised again with ketamine and xylazine, weighed, shaved and echocardiography was performed. Thereafter, animals were sacrificed by cardiac puncture. The heart was removed and snap-frozen.

Cardiac Echocardiography. Forty-eight hours after induction of polymicrobial sepsis, mice were anesthetized (100 mg/kg ketamine and 10 mg/kg xylazine intraperitoneally) and placed on a warming pad (37°C). The thorax was shaved using commercially available hair removal cream. Myocardial performance was measured by echocardiography (2). Heart rate, left ventricular end diastolic diameter (LVEDD), left ventricular end systolic diameter (LVESD), left ventricular end diastolic area (LVEDA) and left ventricular end systolic area (LVESA) were measured. Fractional shortening (FS), fractional area change (FAC) and left ventricular ejection fraction (LVEF) were calculated as followed; FS = (LVEDD-LVESD)/LVEDD; FAC = (LVEDA-LVESA)/LVEDA; EF = (LVEDD3-LVESD3)/LVEDD3.

Histology. Whole hearts from 4 animals/group were stored in 4% formalin and sent for routine staining with Hematoxylin and Eosin (H & E). H&E 6 µm sections (10 per animal) were examined by a single investigator blinded to the treatment status of each animal. The degree of myocardial injury was assessed using an adapted arbitrary myocardial injury scoring system (3) previously published (1), as follows: grade 0, no lesions; grade 1, focal areas of myocardial edema; grade 2, focal lesions extending
over a wider area of myocardial edema associated with cellular gaps and myocardial fiber disruption; grade 3, confluent lesions of myocardial edema, focal areas of necrosis, and cellular infiltration; and grade 4, confluent lesions throughout the heart, gross cellular necrosis, cellular infiltration, fiber disruption and mural thrombi.

**Electron microscopy.** For transmission electron microscopy (TEM), whole heart tissue specimens were fixed overnight at 4 °C in 2.5% glutaraldehyde in Sorensen's phosphate buffer (pH 7.4), osmicated for 1 hour at room temperature in 1% OsO4 in Millonig's buffer (pH 7.4), dehydrated in a graded ethanol series, embedded in an Epon-Araldite mixture, and examined on a Hitachi-7650 transmission electron microscope. For electron microscopy pieces of heart tissue were fixed in 2.5% glutaraldehyde in Sorensen's buffer, postfixed in 1 ~o OsO4 in Millonig's buffer, dehydrated in graded ethanol, and embedded in Epon 812 or in an Epon-Araldite mixture. Semithin sections were cut with an MT-2 ultramicrotome, stained with toluidine blue and examined with an optical microscope to select appropriate areas for electron microscopy. Thin sections were stained with uranyl acetate and lead citrate and studied with a Hitachi-7650 electron microscope.

**Semiquantitative morphological analysis of EM slides.** The scoring method used in this evaluation was adapted from Bishop et al. (4), and included four grades as follows: Grade 0, no evidence of cellular pathology or early autolysis, or an occasional mitochondrion with minimal loss of cristae while the remainder of mitochondria appear normal; Grade 1, discontinuous cristal membranes and/or partial loss of cristae and matrix material in a few mitochondria; Grade 2, multiple disruptions of the cristae membrane and substantial loss of cristae and matrix in approximately half of the mitochondria; Grade 3, fragmented cristal membranes and effacement of central architecture in a majority of the mitochondria. The grading data were not subjected to statistical analysis.

**RNA isolation and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR).** Total RNA from whole hearts was isolated using TRIzol Reagent (Life Technologies, Rockville, MD and Invitrogen, Burlington, Ontario, Canada), and purified using RNAeasy kit (Qiagen, Mississauga, Canada and Qiagen, Chatsworth, CA), as per manufacturer’s specifications. RNA quality was ensured by spectrophometric analysis (OD260/280) and gel visualization. All samples demonstrated good quality cRNA characteristics using Test Probe Array (Affymetrix, Santa Clara, CA). Briefly, a total of 1μg RNA was reverse transcribed to first-strand RNA using the Superscript II system (Invitrogen, Burlington, Canada). The real-time PCR (qRT-PCR) primers were designed using Primer Express (Applied Biosystem I., California, US). The primers used for qRT-PCR are listed in supplemental Table 1. Real-
time PCR was performed by using SYBR Green PCR Master Mix (Perkin Elmer Applied Biosystem Warrington, UK) and amplifying cDNA with an ABI Step-One Plus Sequence Detection System (Applied Biosystem, CA) under universal thermal cycling conditions. Expression was normalised to glyceraldehyde-3- phosphate dehydrogenase (GAPDH) and/or beta-actin (ß-actin). Relative quantity was calculated as previously described (5).

**Microarray Analysis.** Total RNA from whole hearts (collected at 48 h) from 4 animals per group: CLP + vehicle and CLP + RSV was isolated and purified as described (1). High quality cRNA characteristics was determined using Test Probe Array (Affymetrix, Santa Clara, CA), prior to hybridization. A total of 300 ng of mRNA was hybridized to the Illumina Mouse WG 6v1.1 expression bead chip as per manufacturer’s specifications. Illumina raw non-normalised files were uploaded to the R-Project Bioconductor statistical tools package (www.bioconductor.org). Normalised gene expression values were generated for each microarray chip using the Bioconductor package (6), lumi. Variance-stabilizing transformation (VST) method was used to refine normalization (7). Complete array data set and experimental protocol was submitted to the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) according to MIAME standard for microarray data (GSE xxxx). A total of 18,586 probes that passed a one-class analysis in SAM (significant analysis of microarray) were imported into GSEA (8). Changes in gene expression in pathways of interest were visualized using Ingenuity Pathway Analysis (IPA, Ingenuity Systems, Inc. Redwood City, CA).

**Gene Set Enrichment Analysis.** Since the coordinated response to changes in bioenergy metabolism and contractile function, as well as the biologically relevant effects of resveratrol, may be composed of many small cumulative changes in gene expression, we used Gene Set Enrichment Analysis (GSEA, http://www.broad.mit.edu/gsea/index.html) to detect coordinated expression within treated samples of a priori-defined groups of genes (9-11). In contrast to analytical methods based on statistically significant expression changes in a single gene, the GSEA software detects changes in transcriptional activity across the genome by relying on a public database of biologically defined “gene sets”(12). Predefined gene sets may contain genes in a known metabolic pathway, located in the same cytogenetic band, sharing the same Gene Ontology category, or any user-defined parameter. Gene sets are available from Molecular Signatures DataBase (MolSigDB, http://www.broad.mit.edu/gsea/msigdb/msigdb_index.html) (13). GSEA calculates an enrichment score (ES) that reflects the degree to which a gene set is overrepresented at the extremes (top or bottom) of the entire ranked list of microarray data – where genes are ranked according to the expression difference (signal/noise ratio).
between two phenotypes. The ES is calculated by walking down the list, increasing a running-sum statistic when it encounters a gene that is in the gene set and decreasing it when it encounters genes that are not. The magnitude of the increment depends on the correlation of the gene with the phenotype (i.e. CLP + vector or CLP + RSV). The enrichment score is the maximum deviation from zero encountered in the random walk. The software then estimates the statistical significance (nominal P value) of the ES by using an empirical phenotype-based permutation test that preserves the complex correlation structure of the gene expression data. For each permutation the software recomputes the ES, which generates a null distribution for the ES. The empirical, nominal P value of the observed ES is then calculated relative to this null distribution. The permutation of class labels preserves gene-gene correlations and, thus, provides a more biologically robust assessment of significance than would be obtained by permuting genes alone. To adjust the estimated significance level to account for multiple hypothesis testing, GSEA first normalizes the ES for each gene set to account for the size of the set, yielding a normalised enrichment score (NES). It then controls the proportion of false positives by calculating the false discovery rate (FDR) corresponding to each NES. The FDR is the estimated probability that a set with a given NES represents a false positive finding; it is computed by comparing the tails of the observed and null distributions for the NES.

**Selecting Illumina Probes for GSEA.** Before running GSEA, Illumina probe sets were collapsed to one gene level by using the maximum expression value of the probe set in each class and running a One-Class Analysis in SAM (from 46,644 probe sets to 18,586 genes). SAM scores (8) were used to rank the genes. In the one-class analysis probes are scored based on their change in expression relative to the standard deviation of repeated measurements of the probe across all the experiments. Probes with scores greater than a threshold delta are deemed to be significantly changed (irrespective of the absolute fold change). A total of 18,586 genes (One-Class Analysis FDR < 0.9%, delta 7.1888) were used to determine the biological effects of RSV using GSEA. GSEA was run according to default parameters: collapses each probe set into a single gene vector (identified by its HUGO gene symbol), permutation number = 1000, and permutation type = “gene-sets”. By convention a FDR of 25% was used as the cut-off for significance.

**Ingenuity Pathway Analysis.** Analysis of individual differentially expressed genes. We used Significance Analysis of Microarrays (SAM) and Ingenuity Pathway Analysis (IPA, Ingenuity Systems, Inc. Redwood City, CA) as complementary tools to identify individual differentially expressed genes within a dysregulated gene set. We used SAM (http://www-stat.stanford.edu/tibs/SAM/) with a FDR 1.o to
provide a conventional measure of statistical significance for individual differentially expressed genes between classes (8). Functional enrichment analysis was performed by using IPA. By convention genes that were upregulated by RSV (that contribute to the enrichment in gene sets up-regulated by RSV) are shown as red and genes that are down-regulated (contribute to the enrichment in gene sets down regulated by RSV) are shown as green. For IPA analysis, molecules from the data set that are associated with Ingenuity’s Knowledge Base are considered for the analysis. The significance of the association and between the data set and the specific pathways of interest is determined in two ways: i) ratio of the number of molecules from the data set that map to the pathway divided by the total number of molecules that map to the Ingenuity Knowledge Base pathway (ratio) and ii) Fisher’s exact test is used to calculate a p-value determining the probability that the association between the genes in the data set and the pathway of interest can be explained by chance alone (p-value).

**Gene Set Enrichment Analysis of cis-regulatory motifs.** To identify common features amongst RSV regulated genes we used GSEA to screen the 4-kb segment centered on the transcription start site and 3’ region for known transcription factor binding sites contained in the Molecular Signatures DataBase (MolSigDB, C3 data base, (13)). As before, an FDR of <25% is considered statistically significant.

**RNA isolation from tissues and qRT-PCR analysis.** Total RNA was extracted from whole hearts using Trizol reagent (Invitrogen, Burlington, Ontario, Canada) and purified with RNeasy (Qiagen, Chatsworth, CA) as previously described (1,5). RNA quality was ensured by spectrophometric analysis (OD260/280) and gel visualization. High quality cRNA characteristics was determined using Test Probe Array (Affymetrix, Santa Clara, CA), prior to hybridization. Briefly, a total of 1µg RNA was reverse transcribed to first-strand RNA using the Superscript II system (Invitrogen, Burlington, Canada). The real-time PCR (qRT-PCR) primers were designed using Primer Express (Applied Biosystem I., California, US). The primers used for qRT-PCR are listed in supplemental Table 1. Real-time PCR was performed by using SYBR Green PCR Master Mix (Perkin Elmer Applied Biosystem Warrington, UK) and amplifying cDNA with an ABI Step-One Plus Sequence Detection System (Applied Biosystem, CA) under universal thermal cycling conditions. Expression of selected gene(s) was normalised to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and/or beta-actin (β-actin).

**Materials.** PGC-1α, Nrf2 (C-20), β-actin and horseradish peroxidase-conjugated secondary antibodies to mouse or rabbit immunoglobulins were purchased from Santa Cruz Biotech (Santa Cruz, CA). Other chemicals were purchased from Sigma or Fisher Scientific.

**Primary cardiomyocytes isolation.** Primary cultures of neonatal cardiomyocytes were prepared from
Sprague-Dawley rats according to a protocol we previously described (14,15). In brief, cells were disaggregated from heart tissue and differentially plated to remove fibroblasts. Cardiomyocytes were plated in 100-mm Petri dishes at a density of 6 X 10⁶ cells in high glucose Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 1% penicillin/streptomycin. The culture medium was replaced with fresh media for every 48h.

**Western Blot.** Cardiomyocytes incubated in serum-free medium overnight. The cells were treated with LPS (1µg/ml) or Resveratrol (RSV 50 µM) or LPS+RSV or vehicles for 24 hours. Then the cells were lysed with lysis buffer (137 mM NaCl, 20 mM Tris-HCl, pH 7.5, 10% glycerol, 1% Triton X-100, 0.5% Nonidet P-40, 2 mM EDTA, pH 8.0, 3 µg/ml aprotinin, 3 µg/ml leupeptin, 2 mM phenylmethylsulfonyl fluoride, 20 mM NaF, 10 mM NaPP, and 2 mM Na₃VO₄). Equal amounts of proteins from each sample were separated by SDS-PAGE and then transferred to polyvinylidene difluoride membrane and incubated with a blocking buffer (5% nonfat milk in 20 mM Tris-HCl, pH 7.5, 137 mM NaCl, 0.1% Tween 20) for 1 h at room temperature. The membranes were sequentially incubated with primary antibodies overnight at 4 °C, washed three times (20 mM Tris- HCl, pH 7.5, 137 mM NaCl, and 0. 1% Tween 20), incubated with horseradish peroxidase-conjugated secondary antibodies (1:5000 dilution) for 1 h at room temperature, washed three times, and then detected with ECL (Amersham Biosciences).

**Statistics.** No differences were found between the two dosages RSV, data were therefore analyzed collectively. Heart rate data were logarithmically transformed; other data (FS, FAC and EF) were distributed normally. Independent samples t-test were performed. Data are expressed as mean ± standard error of the mean (SEM). A P-value <0.05 was considered significant. Exact P-values are shown.
References


Supplemental Figure 1. A. Survival curves from pilot dose-curve experiment. Survival in the vehicle-treated group (CLP + vehicle = 6) at 48 h was just over 40%. Treatment with ResV (30 [n = 6] or 60 ml/kg [n = 8]) did not lead to a significant improvement in survival. We also investigated the contribution of different dissolvents (vehicle), and confirmed that this did not affect mortality differently (data not shown).

B. Hierarchical clustering of statistically significant physiological variables (FAC, FS, and EF). Individual animal cluster based on treatment parameters. Results are converted to colour, where red indicates increased myocardial contractility (EF³60%), blue decreased contractility (EF≤30%) and grey no change in contractility (EF>30 and <40%).

Supplemental Figure 2. Representative light microscopy slides (HE-staining, 40 and 60x magnification) showing pathological changes in myocardial structure in (i+iv) sham, (ii+v) CLP + vehicle and (iii+vi) CLP + ResV treated mice (n=3/group). Hearts from vehicle treated mice after CLP-induced polymicrobial sepsis showed grade 2-3 injury, i.e. focal lesions and myocardial edema, extending over a wider area of the left ventricle with right ventricular involvement compared to sham where no significant myocardial injury was noted. ResV treated mice developed grade 1-2 injury (focal areas of edema), but were overall spared from development of significant diffuse myocardial edema.
Supplemental Figure 3. Down regulation of genes involved in insulin signaling: A. GSEA enrichment plot for genes involved in insulin signaling (GSEA KEGG pathways, Table 3). As described in Figure 2, top part of each plot shows the progression of the running enrichment score (ES) and the maximum peak therein. The middle part shows the genes in the gene set as “hits” against the ranked list of genes. The lower part shows the histogram for the ranked list of all genes in the expression data set. B. The corresponding heat maps show the expression values for the top subset of genes in each pathway that contributes to the enrichment score in the 8 mice profiled. Results are converted to colour, where red indicates a high and blue a low expression value. False discovery rate (FDR); Gene symbols are described in Supplemental Table 7. C. IPA library of canonical pathways identified insulin signaling as a top pathway altered in murine septic hearts after treatment with ResV (ratio 0.152, p-value 6.96E−13). Figure legend as described in Figure 3.
Chapter 4 - Supplements

Supplemental Figure 4. Down regulation of genes involved in MAPK signaling: A. GSEA enrichment plot for genes involved in MAPK signaling (GSEA KEGG pathways, Table 3). As described in Figure 2, top part of each plot shows the progression of the running enrichment score (ES) and the maximum peak therein. The middle part shows the genes in the gene set as “hits” against the ranked list of genes. The lower part shows the histogram for the ranked list of all genes in the expression data set. B. The corresponding heat maps show the expression values for the top subset of genes in each pathway that contributes to the enrichment score in the 8 mice profiled. Results are converted to colour, where red indicates a high and blue a low expression value. False discovery rate (FDR); Gene symbols are described in Supplemental Table 8. C. IPA library of canonical pathways identified insulin signaling as a top pathway altered in murine septic hearts after treatment with ResV (ratio 0.167, p-value 5.16E^-5). Figure legend as described in Figure 3.
Supplemental Figure 5. Enrichment for genes containing ResV-regulated putative cis-regulatory binding sequences. A. ResV treatment resulted in down regulation of genes known to contain putative Nrf2 (Nuclear factor erythroid-derived 2-like 2, oxidative stress) and NF-kB (nuclear factor kappa-light-chain-enhancer of activated B cells, inflammation/immunity) binding sites. Figure legend described in Figure 3. B. Corresponding heat maps showing the expression values for the top subset of genes in each pathway that contributes to the enrichment score in the 8 mice profiled. C. Leading edge analysis compares top gene sets enriched after treatment with ResV. Graph uses color intensity to show the overlap between subsets: the darker the color, the greater the overlap between the subsets. Intense green cell indicates that horizontal and vertical sets have the same leading edge genes and a white cell indicates that there are no leading edge genes in common. Estrogen-related receptor 1 (ERR1), Forkhead box O3 and 4 (FOXO 3 and 4), Nuclear respiratory factor 1 (Nrf1), CCAAT/enhancer-binding protein (CEBP), Myocyte enhancer factor-2 (MEF2), MyoD (myogenic regulatory factor MyoD), Nuclear factor of activated T-cells (NFAT), cAMP-responsive transcription factor (CREB), Nuclear factor kappa-light-chain-enhancer of activated B cells (NFKB), Activator protein 1 (AP1) and Nuclear factor erythroid-derived 2-like 2 (Nrf2).