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Suppression of Mitochondrial Biogenesis through TLR4-dependent MEK/ERK Signaling in Endotoxin-Induced Acute Kidney Injury

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Nonstandard Abbreviations: MB, mitochondrial biogenesis; AKI, acute kidney injury; ETC, electron transport chain; LPS, lipopolysaccharide; PGC-1α, peroxisome proliferator-activated receptor γ coactivator-1α; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β; MEK, mitogen activated protein kinase kinase; ERK, extracellular signal-regulated kinase; TLR4, toll-like receptor 4; RBF, renal blood flow; IL-6, interleukin 6; CLP, cecal ligation and puncture; BUN, blood urea nitrogen; NRF-1, nuclear respiratory factor-1; TFAM, mitochondrial transcription factor a; NDUFS1, NADH dehydrogenase (ubiquinone) Fe-S protein 1; NDUFB8, NADH dehydrogenase (ubiquinone) beta subcomplex 8; ATPSβ, ATP synthase β; COX1, cytochrome c oxidase subunit 1; ND1, NADH dehydrogenase subunit 1; PBS, phosphate buffered saline; GFR, glomerular filtration rate; KIM-1, kidney injury molecule-1; NGAL, neutrophil gelatinase-associated lipocalin; PGC-1β, peroxisome proliferator-activated receptor γ coactivator-1β; PRC, peroxisome proliferator-activated receptor gamma coactivator-related protein 1; MAPK, mitogen activated protein kinase; JNK, c-Jun N-terminal kinase; TPL-2, tumor progression locus 2; NFκB, nuclear factor κ B; RPTC, renal proximal tubule cell

Recommended Section: Gastrointestinal, Hepatic, Pulmonary, and Renal

Abstract:

Although disruption of mitochondrial homeostasis and biogenesis (MB) is a widely accepted pathophysiological feature of sepsis-induced AKI, the molecular mechanisms responsible for this phenomenon are unknown. In this study, we examined the signaling pathways responsible for the suppression of MB in a mouse model of lipopolysaccharide (LPS)-induced AKI. Down-regulation of PGC-1α, a master regulator of MB, was noted at the mRNA level at 3 h and protein level at 18 h in the renal cortex, and was associated with loss of renal function following LPS treatment. LPS-mediated suppression of PGC-1α led to reduced expression of downstream regulators of MB and electron transport chain (ETC) proteins along with a reduction in renal cortical mitochondrial DNA content. Mechanistically, TLR4 knockout mice were protected from renal injury and disruption of MB after LPS. Immunoblot analysis revealed activation of TPL-2/MEK/ERK signaling in the renal cortex by LPS. Pharmacological inhibition of MEK/ERK signaling attenuated renal dysfunction and loss of PGC-1α, and was associated with a reduction in pro-inflammatory cytokine (e.g. TNF-α, IL-1β) expression at 3 h post-LPS. Neutralization of TNF-α also blocked PGC-1α suppression, but not renal dysfunction, following LPS-induced AKI. Finally, systemic administration of recombinant TNF-α alone was sufficient to produce AKI and disrupt mitochondrial homeostasis. These findings indicate an important role for the TLR4/MEK/ERK pathway in both LPS-induced renal dysfunction and suppression of MB. TLR4/MEK/ERK/TNF-α signaling may represent a novel therapeutic target to prevent mitochondrial dysfunction and AKI produced by sepsis.

Introduction:

Acute kidney injury (AKI) is characterized by a rapid decrease in renal function over the course of hours to days and is associated with significant morbidity and mortality (~40%) (Uchino et al., 2005). Despite recent efforts to better understand AKI, mortality associated with this clinical disorder remains unchanged over the last five decades (Thadhani et al., 1996; Waikar et al., 2008). Sepsis is the most common contributing factor to the development AKI, mortality resulting from AKI is almost doubled in septic patients (~70%) and treatment is limited to dialysis and supportive care (Silvester et al., 2001; Schrier and Wang, 2004; Uchino et al., 2005; Bagshaw et al., 2007; Waikar et al., 2008). Taken together, these data reveal a significant need for further study of the pathophysiological mechanisms underlying renal injury with an emphasis on identifying therapeutic targets to improve clinical outcomes in septic AKI.

Much of the difficulty in developing effective therapies for sepsis-induced AKI stems from the multi-factorial nature of the disease. Septic AKI is thought to arise as a result of complex interactions involving alterations in renal hemodynamics, microvascular/endothelial cell dysfunction and direct effects of inflammatory cells and their products (cytokines/chemokines) on the kidney (Wan et al., 2008). The degree to which changes in global renal blood flow (RBF) contribute to renal injury remains a topic of intense debate. However, it is generally accepted that microvascular dysfunction leads to sluggish capillary flow and subsequent development of local regions of hypoperfusion and hypoxia in the septic kidney (Wu et al., 2007a; Wu et al., 2007b; Gomez et al., 2014). Reduced microvascular flow also amplifies injury by prolonging exposure of the renal parenchyma to inflammatory cells and various inflammatory molecules including pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and IL-6, which are primary mediators of cellular injury in

sepsis-induced AKI (Cunningham et al., 2002; Chawla et al., 2007; Wang et al., 2011; Xu et al., 2014). Data from post-mortem studies and experimental models indicate that tubular cell apoptosis and necrosis are relatively limited in the septic kidney when compared to other forms of AKI (Guo et al., 2004; Langenberg et al., 2008). However, histological findings including tubular cell vacuolization, tubular dilatation, and the presence of swollen mitochondria provide strong evidence that sub-lethal injury to the proximal tubule may play an important role in the development of sepsis-induced AKI (Langenberg et al., 2008; Tran et al., 2011; Takasu et al., 2013).

The overt structural changes noted in tubular mitochondria following sepsis-induced AKI is of particular note given that the proximal tubule relies heavily on mitochondrial generation of ATP to drive active transport of electrolytes and fluids. Thus, mitochondrial and/or proximal tubule dysfunction may contribute to loss of renal function and disease progression in AKI (Soltoff, 1986; Thadhani et al., 1996). Recent studies demonstrated suppression of peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) and consequently MB in experimental models of sepsis-induced AKI including systemic endotoxin exposure and cecal ligation and puncture (CLP) (Tran et al., 2011). PGC-1α, a "master regulator of MB," promotes transcriptional activity in both the nucleus and mitochondria to facilitate generation of new, functional mitochondria in response to a variety of physiological stimuli (Finck and Kelly, 2006; Sanchis-Gomar et al., 2014). Loss of PGC-1α following sepsis-induced AKI was closely associated with renal and mitochondrial dysfunction and reduced expression of electron transport chain proteins. In addition, proximal tubule-specific PGC-1α knockout delayed recovery of renal function following saline resuscitation in mice treated with LPS (Tran et al., 2011). These

findings indicate that suppression of PGC-1α and MB may play an important role in disease progression and recovery in the setting of septic AKI.

The aim of the current study was to determine the signaling mechanisms responsible for suppression of MB in the renal cortex following endotoxic AKI. We report that LPS exposure leads to down-regulation of PGC-1α and mitochondrial markers in the renal cortex. LPS-induced renal dysfunction and disruption of MB was dependent on TLR4/MEK/ERK and production of the pro-inflammatory cytokine TNF-α. Inhibition of the TLR4/MEK/ERK/TNF-α signaling may offer a novel therapeutic approach to reverse suppression of MB and loss of renal function in septic AKI.

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Materials and Methods:

LPS Model of Sepsis-Induced AKI

Six to eight week old male C57BL/6 mice were acquired from the National Institutes of Health National Cancer Institute / Charles River Laboratories (Frederick, MD). Mice were given an intraperitoneal (i.p.) injection of 0.5, 2 or 10 mg/kg lipopolysaccharide (LPS) derived from *Escherichia coli* serotype O111:B4 (Sigma Aldrich, St. Louis, MO). Control mice received an i.p. injection of an equal volume of 0.9% normal saline. Mice were euthanized by isoflurane asphyxiation and cervical dislocation at 1, 3, and 18 h after LPS administration, and kidneys and serum were collected for molecular analysis. For experiments utilizing TLR4-deficient animals, TLR4KO mice were generated by crossing C57BL/10ScN mice with the $tlr4^{LPS\text{-}d}$ mutation onto the C57BL/6 background for at least five generations (Ellett et al., 2009). All studies were conducted in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Animal use was approved by the Institutional Animal Care and Use Committee at the Medical University of South Carolina.

To determine the role of MEK/ERK signaling in LPS-induced AKI, the MEK inhibitor GSK1120212 (trametinib, chemical structure provided in (Gilmartin et al., 2011)) was obtained from Selleckchem Chemicals (Houston, TX). GSK1120212 is a potent and specific inhibitor of MEK1/2 which has been previously used in mouse models (Yamaguchi et al., 2011; Yamaguchi et al., 2012). Mice received an i.p. injection of GSK1120212 (1 mg/kg) or vehicle control (DMSO) 1 h prior to administration of LPS.

To assess the effects of TNF-α on regulation of MB in this model, rat anti-TNF-α neutralizing antibody (clone MP6-XT22) and the appropriate rat IgG1 κ isotype control antibody (clone RTK2071) were purchased from BioLegend (San Diego, CA). Mice were randomly

assigned to one of three groups: 1) control, 2) LPS + isotype control antibody (25 mg/kg), and 3) LPS + anti-TNF-α neutralizing antibody (25 mg/kg). Isotype control antibody and anti-TNF-α neutralizing antibody were administered intravenously (i.v.) 1 h prior to LPS via tail vein injection. Control mice received an i.v. injection of vehicle control (PBS).

Recombinant mouse TNF-α was obtained from BioLegend to evaluate whether TNF-α alone reproduced LPS-mediated changes in renal function and/or MB. Wild-type C57BL/6 male mice (6 to 8 weeks in age) were treated i.v. with either vehicle control (diluent), 20 μg/kg recombinant murine TNF-α, or 50 μg/kg TNF-α via tail vein injection. Animals were euthanized at 18 h after TNF-α administration and kidneys and serum were collected for analysis.

Blood Urea Nitrogen Measurement

Blood urea nitrogen (BUN) was determined using the QuantiChrom Urea Assay kit (BioAssay Systems, Hayward, CA) based on the manufacturer's directions. All values are expressed as blood urea nitrogen concentration in milligrams per deciliter.

Quantitative Real-Time PCR Analysis of mRNA Expression

Total RNA was isolated from renal cortical tissue with TRIzol reagent (Life Technologies, Grand Island, NY). The iScript Advanced cDNA Synthesis Kit for RT-qPCR (Bio-Rad, Hercules, CA) was used to produce a cDNA library from 1 μg total RNA according to the manufacturer's protocol. Quantitative real-time PCR was performed with the generated cDNA using the SsoAdvanced Universal SYBR Green Supermix reagent (Bio-Rad). Relative mRNA expression of all genes was determined by the $2^{-\Delta\Delta Ct}$ method and the 18S ribosomal RNA

(18S rRNA) was used as a reference gene for normalization as previously described (Wills et al., 2012). Primer pairs used for PCR were as described in Table 1.

Analysis of Mitochondrial DNA Content

Mitochondrial DNA content was determined by quantitative real-time PCR analysis. Total DNA was isolated from the renal cortex using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) as described in the manufacturer's protocol. Extracted DNA was quantified and 5 ng was used for PCR. Relative mitochondrial DNA content was assessed by the mitochondrial-encoded NADH Dehydrogenase 1 (ND1) and was normalized to nuclear-encoded β-Actin. Primer sequences for ND1 and β-Actin were: ND1 sense: 5'-TAGAACGCAAAATCTTAGGG-3'; ND1 antisense: 5'-TGCTAGTGTGAGTGATAGGG-3'; β-Actin sense: 5'-GGGATGTTTGCTCCAACCAA-3'; and β-Actin antisense: 5'-GCGCTTTTGACTCAGGATTTAA-3'.

Immunoblot Analysis

Protein was extracted from renal cortex using RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, pH 7.4) with protease inhibitor cocktail (1:100), 1 mM sodium fluoride, and 1 mM sodium orthovanadate (Sigma Aldrich). Total protein amount was determined by BCA protein assay. Equal protein quantities (50 – 100 μg) were loaded onto 4 – 15% SDS-PAGE gels (Bio-Rad). Proteins were resolved by gel electrophoresis and transferred onto nitrocellulose membranes (Life Technologies). Membranes were blocked in 2.5% BSA and incubated overnight with primary antibody at 4°C. Primary antibodies used in these studies included NGAL/Lipocalin-2 (1:1000), phospho-TPL2 (1:500), total TPL2 (1:1000, all from Abcam, Cambridge, MA), phospho-ERK1/2 (1:1000), total ERK1/2

(1:1000, both from Cell Signaling Technology, Danvers, MA), KIM1 (1:1000, from R&D systems, Minneapolis, MN), PGC-1 (1:100, Cayman Chemical, Ann Arbor, MI), and β-Actin (1:1000, Santa Cruz Biotechnology, Dallas, TX). Membranes were incubated with the appropriate horseradish peroxidase(HRP)-conjugated secondary antibody before visualization using enhanced chemiluminescence (Thermo Scientific, Waltham, MA) and the GE ImageQuant LAS4000 (GE Life Sciences, Pittsburgh, PA). Optical density was determined using NIH ImageJ software (version 1.46).

Statistical Analysis

All data are shown as mean ± S.E.M. When comparing two experimental groups, an unpaired, two-tailed t-test was used to determine statistical differences. A one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test was performed for comparisons of multiple groups. A p-value < 0.05 was considered statistically significant. All statistical tests were performed using GraphPad Prism software.

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Results:

Endotoxin Exposure Leads to Acute Kidney Injury in Mice

To confirm the effects of systemic LPS exposure on renal function, we measured BUN at 3 and 18 h after administration in mice. BUN increased 1.9-fold and 3.8-fold 3 h and 18 h post-LPS, respectively, indicating a time-dependent reduction in renal function (Figure 1A). These findings are consistent with other studies reporting markedly reduced glomerular filtration rate (GFR) and elevated BUN with 4 h of endotoxin exposure in mice (Bhargava et al., 2013). To confirm proximal tubule injury in this model, we also measured protein expression of kidney injury molecule-1 (KIM-1) and neutrophil gelatinase-associated lipocalin (NGAL) in the renal cortex by immunoblot analysis at 18 h. KIM-1 and NGAL increased ~5- and ~2-fold, respectively, in mice receiving LPS when compared to vehicle-treated controls (Figure 1B,C). No changes were noted in KIM-1 and NGAL protein levels at 3 h post-LPS, although their mRNAs were up-regulated at this time point (data not shown). Together, these findings indicate that systemic LPS exposure results in rapid development of AKI and specifically leads to injury in the renal proximal tubule.

LPS-Induced AKI Leads to Persistent Suppression of PGC-1α

Because disruption of MB and homeostasis has been associated with loss of renal function and delayed recovery in multiple experimental models of AKI, we hypothesized that systemic endotoxin exposure would lead to disruption of PGC-1α. (Tran et al., 2011; Funk and Schnellmann, 2012; Funk and Schnellmann, 2013). PGC-1α mRNA was reduced by 70% as early as 3 h after LPS and progressively decreased at the 18 h time point to ~10% of control (Figure 2A). In addition, a ~70% decrease in PGC-1α protein expression was noted at 18 h after

LPS exposure (Figure 2B). No change was observed in PGC-1α protein at the 3 h time point (data not shown). To determine whether LPS has a dose-dependent effect on renal cortical PGC-1α expression, mice were treated with 0.5 mg/kg and 2 mg/kg of LPS and PGC-1α mRNA was examined 3 h after administration. Interestingly, lower doses of LPS (0.5 mg/kg and 2 mg/kg) reduced PGC-1α transcript ~55% and ~45%, respectively, similar to the 10 mg/kg dose (~60%) (Figure 2C).

To further characterize the effects of endotoxin on MB, we measured mRNA expression of other members of the PGC-1 family, PGC-1β and PRC, in the renal cortex. Transcript levels of PGC-1β were decreased ~60% in LPS-treated mice at 3 and 18 h compared to vehicle-treated controls. In contrast, PRC mRNA increased 1.7-fold as early as 3 h post-administration and remained elevated at 18 h (2-fold) (Figure 2D). These data demonstrate that systemic LPS exposure results in suppression of PGC-1α and PGC-1β at the mRNA and/or protein levels in the renal cortex.

PGC-1α Suppression by LPS Results in Disruption of Mitochondrial Homeostasis

To determine whether mitochondrial homeostasis was altered in the renal cortex as a result of reduced PGC-1α expression following endotoxin administration, we assessed mRNA expression of downstream mediators of MB, (NRF-1, TFAM) as well as nuclear-encoded (NDUFS1, NDUFB8, ATPSβ) and mitochondrial-encoded (COX1, ND1) components of the electron transport chain. A ~38% decrease in NRF-1 mRNA was observed 3 h post-LPS which recovered to control levels by 18 h. Transcript levels of TFAM, NDUFS1, NDUFB8, ATPSβ, and COX1 were also decreased (~25%, 43%, 42%, 25%, and 31%, respectively) at the 18 h time-point and these decreases correspond with the reduction in PGC-1α protein (Figure 3A).

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We also assessed mitochondrial DNA copy number as a marker of mitochondrial quantity. Relative mitochondrial DNA content in the renal cortex was reduced 43% 18 h after LPS (Figure 3B). These findings indicate that suppression of PGC-1α following systemic endotoxin exposure is associated with widespread disruption of transcriptional regulation of mitochondrial proteins and depletion of mitochondrial DNA content.

TLR4 is Required for Endotoxin-Induced AKI and Disruption of Mitochondrial Homeostasis

Although LPS primarily signals through activation of Toll-like receptor 4 (TLR4), recent studies have identified TLR4-independent signaling pathways which contribute to endotoxic shock in mice (Hagar et al., 2013; Kayagaki et al., 2013). To determine whether TLR4 signaling is responsible for both endotoxin-induced AKI and suppression of MB, we utilized TLR4 knockout mice (TLR4KO) and wild-type controls and assessed renal function and mitochondrial homeostasis 18 h post-LPS. As expected, systemic LPS exposure in wild-type mice resulted in an increase in BUN (Figure 4A). However, TLR4KO mice were completely protected from endotoxic AKI. These findings are in agreement with previous studies demonstrating an essential role for TLR4 in LPS-induced renal dysfunction in mice (Cunningham et al., 2004).

We next determined the role of TLR4 in endotoxin-mediated suppression of PGC-1α and MB. A 73% decrease in PGC-1α mRNA was noted in wild-type mice subjected to LPS while PGC-1α mRNA in TLR4KO mice was similar to controls (Figure 4B). COX1 mRNA and mitochondrial DNA content were also decreased (~40% and ~30%, respectively) in wild-type mice 18 h after LPS exposure and remained unchanged in TLR4KO mice (Figure 4C,D). Taken together, these data reveal an essential role for TLR4 in both LPS-mediated renal damage and suppression of MB.

LPS-Induced AKI Leads to Activation of TPL-2/ERK Signaling in the Renal Cortex

TLR4 is known to initiate signaling by a number of different mediators, most notably NFκB and members of the mitogen-activated protein kinase (MAPK) family including extracellular signal-related kinases 1/2 (ERK1/2), p38 MAPKs, and c-Jun N-terminal kinases (JNKs) (Ostuni et al., 2010; Lin and Tang, 2014). Previous studies showed that ERK1/2 activation may negatively regulate expression of PGC-1α in multiple organs including the brain and skeletal muscle (Coll et al., 2006; Ashabi et al., 2012). In addition, TPL-2 is thought to be a critical for activation of ERK1/2 in response to LPS (Dumitru et al., 2000; Banerjee et al., 2006). We hypothesized that the TPL-2/ERK signaling would be activated in the renal cortex and that this pathway contributes to disruption of MB in endotoxin-induced AKI. Phosphorylated TPL-2 (Thr209) increased 1.5-fold 1 h after LPS treatment and returned to control levels at 3 h (Figure 5). A 5-fold elevation of ERK1/2 phosphorylation was observed 1 h after LPS treatment and remained elevated at 3 h (Figures 5B and 6D). These findings indicate that TPL-2/ERK signaling is activated quickly in the renal cortex in response to systemic endotoxin exposure.

Early ERK Activation Mediates LPS-Induced Renal Injury and Suppression of MB

To further elucidate the role of ERK1/2 signaling in LPS-induced AKI and disruption of mitochondrial homeostasis, mice were treated with the potent and selective MEK1/2 inhibitor GSK1120212 (GSK112) (1 mg/kg) 1 h prior to LPS administration. MEK1/2 are directly responsible for phosphorylation of ERKs and thus inhibition of MEK1/2 by GSK112 will prevent downstream activation of ERK1/2. In the first experiment, mice were euthanized 3 h post-LPS to determine the early effects of ERK activation on renal dysfunction and MB. Pre-treatment with GSK112 attenuated endotoxin-mediated elevation of BUN at 3 h (Figure 6A) and

markedly reduced the increase in renal cortical KIM-1 transcript levels in LPS-treated mice (Figure 6B). GSK112 alone did not have an effect on either BUN or renal cortical KIM-1 (Figures 6A and 6B). Interestingly, GSK112 administration in mice receiving LPS also blocked suppression of PGC-1α mRNA expression (Figure 6C). Immunoblot analysis demonstrated effective blockade of ERK1/2 activation in mice treated with MEK1/2 inhibitor (Figure 6D). These data identify a novel role for early TLR4-mediated ERK1/2 activation in both renal dysfunction and suppression of MB in the renal cortex in LPS-induced AKI.

A separate group of mice was pretreated with GSK112 (1 mg/kg) or vehicle control and serum and kidneys were collected at 18 h post-LPS for biochemical analysis. BUN increased 3.6-fold in LPS-treated animals and was partially attenuated by GSK112 (~2.1 fold vs. control) (Figure 7A). In addition, GSK112 pretreatment partially blocked LPS-induced increase in renal cortical KIM-1 mRNA expression, indicating a reduction in tubular injury (Figure 7B). A modest effect of GSK112 administration on transcript levels of PGC-1α and its transcriptional target NDUFS1 was also noted in LPS mice (Figures 7C and 7D). Taken together, these findings indicate that rapid activation of ERK1/2 in the renal cortex following systemic endotoxin exposure contributes to the early development of renal injury and disruption of mitochondrial homeostasis.

Early ERK Activation Promotes Expression of Pro-Inflammatory Cytokines in the Renal Cortex After LPS Administration

TLR4-mediated activation of ERK1/2 is known to induce expression of pro-inflammatory cytokines including TNF-α and IL-1β (DeFranco et al., 1998; Schaefer et al., 2005; Luan et al., 2014). Recent reports also indicate that these cytokines may negatively regulate PGC-1α

expression and MB in a variety of cell types including renal proximal tubule cells and skeletal muscle cells (Tran et al., 2011; Remels et al., 2013). We therefore examined ERK1/2 signaling in the expression of TNF-α and IL-1β in the renal cortex following systemic endotoxin administration in mice. LPS administration resulted in a robust increase in TNF-α (~12 fold) and IL-1β (~3.3 fold) mRNA at 3 h and GSK112 inhibition of MEK/ERK partially attenuated the early increase in mRNA expression of both pro-inflammatory cytokines (Figure 8A). However, pretreatment with GSK112 had no effect on expression of either TNF-α or IL-β at 18 h post-LPS (Figure 8B). These findings reveal an important role for TLR4-induced ERK signaling in early induction of pro-inflammatory cytokines in the renal cortex after systemic endotoxin administration.

Involvement of TNF-α in LPS-Mediated Suppression of PGC-1α

Because pro-inflammatory cytokines have been implicated as a potential mechanism for suppression of PGC-1α in renal proximal tubules and ERK signaling promoted TNF-α expression in the kidney cortex, we utilized a neutralizing antibody directed against murine TNF-α to further elucidate its role in this process. Mice were treated i.v. with either TNF-α neutralizing antibody (25 mg/kg) or the appropriate isotype control antibody (25 mg/kg) 1 h prior to LPS. TNF-α neutralization had no effect on the LPS-induced increase in BUN at 18 h (Figure 9A). However, suppression of PGC-1α mRNA levels was partially attenuated in mice receiving the anti-TNF-α antibody (Figure 9B).

Surprisingly, TNF-α neutralization had no effect on the observed decreases in NDUFB8, NDUFS1, ATPSβ, and COX1 transcripts, and mitochondrial DNA content (Figure 9C,D).

Taken together, these data indicate that the TLR4/ERK/TNF-α signaling axis may act as a critical mechanism for suppression of MB in the setting of endotoxic AKI.

Recombinant TNF-α is Sufficient to Produce Kidney Injury and Disrupt MB in the Renal Cortex

To further characterize the effects of TNF-α on renal dysfunction and mitochondrial homeostasis, mice received i.v. injections of low dose recombinant murine TNF-α (20 μg/kg), high dose TNF-α (50 μg/kg), or the appropriate vehicle. TNF-α dosing was based on preliminary experiments and previous reports to determine the appropriate doses which have no effect on renal function (low dose) or produce renal injury (high dose) (Xu et al., 2014). As expected, an increase in BUN was noted only in mice receiving high dose TNF-α (Figure 10A). Renal dysfunction in these animals was associated with a 55% decrease in renal cortical PGC-1α mRNA (Figure 10B). Both low and high dose TNF-α suppressed transcript levels of both nuclear-encoded (NDUFS1) and mitochondrial-encoded (ND1, COX1) respiratory proteins (Figure 10C). A modest, but not statistically significant, decrease in mitochondrial DNA content was observed in animals treated with high dose TNF-α (Figure 10D). These findings indicate that recombinant TNF-α is sufficient to cause both renal injury and suppression of MB in the renal cortex.

Discussion:

Disruption of mitochondrial homeostasis is an important contributor to initiation and progression of both tubular injury and renal dysfunction in AKI (Tran et al., 2011; Funk and Schnellmann, 2012; Che et al., 2014). Mitochondrial turnover is regulated by a complex interplay between fission/fusion, autophagy/mitophagy, and MB (Gottlieb and Gustafsson, 2011; Stallons et al., 2013). Our laboratory and others have recently demonstrated alterations in MB in experimental models of AKI (Weinberg, 2011; Funk and Schnellmann, 2012; Jesinkey et al., 2014; Stallons et al., 2014). MB is tightly controlled by the PGC-1 family of transcriptional co-activators including PGC-1α, PGC-1β, and PRC which coordinate both nuclear and mitochondrial responses to increase cellular mitochondrial content. PGC-1α is considered a "master regulator" of MB in organs with high energy demand including the kidney (Puigserver et al., 1998; Scarpulla, 2011) PGC-1α expression is localized to renal proximal tubule cells which rely on mitochondrial ATP production to facilitate active transport of solutes, indicating that MB may be essential for tubular function (Portilla et al., 2002).

Recent studies characterized mitochondrial dysfunction in sepsis-induced AKI (Tran et al., 2011; Patil et al., 2014). Tran et al. demonstrated that mitochondrial dysfunction was associated with acute down-regulation of PGC-1α in the kidney following endotoxin exposure or cecal ligation and puncture (CLP) in mice (Tran et al., 2011). PGC-1α suppression in these models was correlated with both reduced expression of transcriptional targets of PGC-1α and renal dysfunction. Restoration of normal renal function in LPS-treated mice given saline resuscitation at the 18 h time point coincided with return of PGC-1α mRNA and protein to baseline levels, further indicating a potential role for MB in both pathogenesis of and recovery

from septic AKI. Proximal tubule-specific PGC-1α knockout in mice resulted in prolonged renal dysfunction in response to systemic LPS exposure. Together, these findings provide strong evidence that PGC-1α and MB may be viable therapeutic targets in sepsis-induced AKI.

Systemic endotoxin exposure in mice led to rapid deterioration of renal function as measured by BUN at 3 h, and further increases in BUN and renal cortical KIM-1 and NGAL at 18 h. Despite relative minimal renal histological findings, these data provide strong molecular evidence for involvement of tubular insult in the pathophysiology of sepsis-induced AKI.

Progressive renal dysfunction and tubular cell injury were closely associated with a reduction in PGC-1α mRNA and protein in the renal cortex after endotoxin administration. These findings are similar to previous studies reporting negative correlations between BUN and PGC-1α transcript levels in mice receiving LPS or CLP (Tran et al., 2011). Interestingly, lower doses of LPS that did not induce renal dysfunction acutely had a similar effect on PGC-1α mRNA and PGC-1α protein levels did not change at the 3 h time point when renal and tubular injury were first observed as indicated by increased BUN and KIM-1 mRNA expression. These findings indicate that suppression of PGC-1α and subsequent MB is unlikely to be the initial causative factor and alone is not sufficient for the development of sepsis-induced AKI.

Early increases in circulating pro-inflammatory cytokines and renal microvascular dysfunction are also important mediators of renal injury in mice following systemic LPS exposure (Cunningham et al., 2002; Wu et al., 2007b). However, suppression of renal cortical PGC-1α and MB likely contribute to disease progression through disruption of energy-dependent tubular transport and repair processes (Soltoff, 1986; Thadhani et al., 1996; Nowak et al., 1998). This idea is supported by earlier studies demonstrating that both global and proximal-tubule

specific PGC-1α knockout leads to persistent renal injury in LPS-induced AKI in mice (Tran et al., 2011).

We also measured transcript levels of PGC-1β and PRC. PGC-1β expression was decreased, but not as markedly as PGC-1α. In contrast, PRC mRNA levels rose after LPS treatment similar to findings reported in I/R- and glycerol-induced AKI (Funk and Schnellmann, 2012). Given the relative lack of knowledge regarding the role of PGC-1β and PRC in renal mitochondrial homeostasis, it is difficult to interpret these findings and further work is warranted.

Previous studies showed that TLR4 is required for LPS-induced renal dysfunction (Cunningham et al., 2004). However, a number of TLR4-independent actions of LPS have been described (Hagar et al., 2013; Kayagaki et al., 2013). As predicted, TLR4-deficient mice did not develop renal injury and markers of MB and mitochondrial DNA content did not decrease following endotoxin exposure. These findings demonstrate that TLR4 is required for suppression of renal PGC-1α and MB in LPS-induced AKI. It is important to note that the MB response to sepsis is tissue-specific. Up-regulation of hepatic PGC-1α and PGC-1β is markedly enhanced in TLR4-deficient mice after sepsis, suggesting that TLR4 may be an important negative regulator of MB (Sweeney et al., 2010). Further studies are needed to evaluate the possibility that TLR4 activation is a common mechanism underlying suppression of MB in multiple forms of AKI.

Binding of LPS to TLR4 activates a number of downstream signaling mediators, most notably nuclear factor kappa B (NFκB) and mitogen-activated protein kinases (MAPKs) including ERK1/2, p38 MAPKs, and JNKs (Ostuni et al., 2010; Lin and Tang, 2014). We

observed rapid (1 h) activation of TPL-2/MEK/ERK signaling in the renal cortex. These findings are similar to previous studies demonstrating TLR4-dependent increases in ERK1/2 phosphorylation after LPS exposure in the medullary thick ascending limb of the kidney (Watts et al., 2013). Although TPL-2 is known to be required for LPS-induced ERK1/2 activation in macrophages, this is the first study to demonstrate increased TPL-2 phosphorylation in the renal cortex in response to systemic endotoxin exposure (Dumitru et al., 2000; Banerjee et al., 2006). A recent study did reveal that TPL-2 knockout mice are protected from kidney I/R, although this effect was not attributed to the MAPK function of TPL-2 (Yaomura et al., 2008).

MEK/ERK signaling has been implicated as a negative regulator of both PGC-1α and MB. Pharmacological inhibition of MEK reversed reductions in protein levels of PGC-1α and downstream regulators of MB (NRF-1, TFAM) in the hippocampi of rats receiving intracerebroventricular injections of amyloid β (Ashabi et al., 2012). In addition, blockade of ERK activation prevented palmitate-induced down-regulation of PGC-1α in skeletal muscle myotubes *in vitro* (Coll et al., 2006). The MEK1/2 inhibitor GSK1120212 (GSK112) blocked ERK1/2 activation and reduced early renal dysfunction and tubular injury (3 h) in mice subjected to systemic LPS. The protective effects of GSK112 were associated with restoration of renal cortical PGC-1α expression. These findings indicate an important role for MEK/ERK signaling in LPS-induced renal injury and suppression of MB. In mice followed to 18 h post-LPS, GSK112 partially attenuated loss of renal function and injury to the proximal tubule. However, only modest effects were noted on PGC-1α expression and mitochondrial DNA content. This may indicate that the TPL-2/MEK/ERK pathway is essential for early suppression of PGC-1α in the renal cortex after endotoxic AKI, but other signaling mechanisms contribute to late-phase disruption of MB and renal dysfunction. Among these, NFκB has been identified as a potential

regulator of PGC-1α transcriptional activity (Alvarez-Guardia et al., 2010; Remels et al., 2013). Further work is necessary to address the role of NFκB in LPS-induced mitochondrial dysfunction.

MEK/ERK activation downstream of TLR4 results in activation of a transcriptional program to increase cytokine expression in response to LPS (Guha et al., 2001; Banerjee et al., 2006). In addition, pro-inflammatory cytokines have been implicated in regulation of PGC-1α and MB (Tran et al., 2011; Remels et al., 2013). Administration of exogenous TNF-α reduced expression of PGC-1α and its transcriptional targets in primary human renal epithelial cells *in vitro* (Tran et al., 2011). In addition, TNF-α and IL-1β induced disruption of MB in skeletal muscle cells (Remels et al., 2013). As expected, inhibition of MEK/ERK signaling attenuated early up-regulation of both TNF-α and IL-1β in the renal cortex of LPS-treated mice. Mice pretreated with an anti-TNF-α neutralizing antibody and exposed to LPS exhibited less PGC-1α mRNA loss. The partial restoration of PGC-1α following TNF-α neutralization may indicate that multiple pro-inflammatory cytokines (TNF-α, IL-1β, IL-6) signal through similar mechanisms to suppress PGC-1α in the setting of sepsis-induced AKI. To our knowledge, this is the first study to report the effects of TNF-α neutralization on PGC-1α and subsequent MB *in vivo*. We did not detect an effect of anti-TNF-α on BUN 18 h post-LPS. However, an earlier study using the same TNF-α neutralizing antibody before endotoxin administration noted a significant reduction in BUN at the 4 h time point (Bhargava et al., 2013). However, we were able to reproduce the effects of LPS on renal dysfunction and suppression of PGC-1α expression by administration of recombinant TNF-α in naïve mice. Together, these findings strongly implicate TNF-α as a

downstream mediator of TLR4/MEK/ERK signaling in LPS-induced AKI and PGC-1α and MB suppression.

A significant decrease in cortical peritubular capillary flow has been noted as early as 2 h post-LPS exposure in mice indicating that LPS has rapid effects on renal hemodynamics (Wu et al., 2007b). A limitation of the current study is that it does not address whether modulation of TLR4/MEK/ERK and TNF-α signaling might improve microvascular function and thereby restore glomerular filtration in response to LPS. TNF-α neutralization using the soluble decoy receptor TNFsRp55 has been shown to restore both glomerular filtration and renal plasma flow after systemic exposure to a lower dose of LPS (5 mg/kg) (Knotek et al., 2001). Future studies using conditional, tissue-specific knockout TLR4 and downstream signaling targets in the tubular epithelium, vascular endothelium, and vascular smooth muscle may address whether the renal protective effects observed in this study are due to direct actions of pharmacological agents on the tubular epithelium or indirect actions on the renal vasculature.

The current study identifies a novel mechanism mediating injury and suppression of MB in a mouse model of septic AKI. In particular, the TLR4/TPL-2/MEK/ERK/TNF-α signaling axis was required for early renal dysfunction and disruption of mitochondrial homeostasis in LPS-induced AKI. This pathway may represent a viable therapeutic target in the treatment of sepsis-associated AKI. Further studies are warranted to determine whether this mechanism is common to other forms of AKI and to identify the downstream targets of ERK and inflammatory cytokines in this process.

Authorship Contributions:

Participated in research design: Smith, Stallons, Collier, Schnellmann.

Conducted experiments: Smith, Stallons, Collier.

Contributed new reagents or analytic tools: Chavin.

Performed data analysis: Smith, Stallons, Collier, Schnellmann.

Wrote or contributed to the writing of the manuscript: Smith, Schnellmann.

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Footnotes:

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Figure Legends:

Figure 1: Systemic LPS administration induces renal dysfunction and tubular injury in mice. Six to eight week old male C57BL/6 mice were injected i.p. with lipopolysaccharide (10 mg/kg) or saline vehicle and euthanized at 3 and 18 h. Renal function at 3 and 18 h after LPS was determined by BUN (n = 7 animals / group) (**A**). Immunoblot analysis of renal cortical KIM-1 (**B**) and NGAL (**C**) expression was used to assess tubular injury (n = 4 animals / group). Data are shown as mean ± S.E.M. for each group. Different superscripts above bars indicate statistically significant differences ($p < 0.05$) when compared to time-point controls.

Figure 2: LPS-induced AKI is associated with suppression of PGC-1α expression in the renal cortex. Total RNA and protein were extracted from the renal cortex of vehicle- and LPS-treated mice. Time-dependent changes in PGC-1α mRNA expression at 1, 3, and 18 h after LPS exposure (10 mg/kg) were determined by qPCR analysis (n = 5 – 6 / group) (**A**). Protein levels of PGC-1α were assessed by Western blot (n = 4 / group) (**B**). To determine dose dependent effects of endotoxin on renal cortical PGC-1α expression, a separate group of mice were treated with varying doses of LPS (0.5 mg/kg, 2 mg/kg, and 10 mg/kg) and PGC-1α mRNA was measured (n = 6 / group) (**C**). Time-dependent changes in other members of the PGC-1 family (PGC-1β, PRC) were also determined by qPCR (n = 6 / group) (**D)**. Values are expressed as relative expression compared to time-point controls and are presented as mean ± S.E.M. Statistically significant differences ($p < 0.05$) are denoted by superscripts above bars.

Figure 3: Disruption of mitochondrial homeostasis in endotoxic AKI. Total RNA and DNA were harvested from renal cortical tissue of mice treated with saline vehicle or LPS (10 mg/kg, i.p.) at 1, 3, 18, and 42 h. Expression of key regulators of mitochondrial biogenesis (NRF-1,

TFAM), nuclear-encoded components of the ETC (NDUFS1, NDUFB8, ATPSβ), and mitochondrial-encoded respiratory proteins (COX1, ND1) were measured at the mRNA level (n = 6 / group) (**A**). Relative mitochondrial DNA content in the renal cortex was determined by qPCR analysis (n = 5 – 6 animals / group) (**B**). Data are shown as expression relative to vehicle-treated animals (mean ± S.E.M). In panel A, *p < 0.05 vs. time point controls. In panel B, superscripts over bars indicate statistically significant differences between groups (p < 0.05) when compared to time-point controls.

Figure 4: TLR4 is necessary for development of LPS-induced AKI and suppression of mitochondrial biogenesis. Wild-type and TLR4 knockout mice were treated with LPS (10 mg/kg, i.p.) or saline vehicle and euthanized at 18 h for biochemical analysis. BUN was used to assess renal function after LPS exposure (**A**). Transcript levels of PGC-1α (**B**) and COX1 (**C**) as well as mitochondrial DNA copy number (**D**) served as markers of mitochondrial homeostasis (n = 3 – 4 / group for all analyses, p < 0.05 vs. wild-type control).

Figure 5: Systemic LPS exposure enhances TPL-2/ERK signaling in the renal cortex. Mice were treated with LPS (10 mg/kg, i.p.) or vehicle as described above. Immunoblot analysis revealed increased phosphorylated/activated TPL-2 (Thr209) (**A**) and ERK1/2 (Thr202/Tyr204) (**B**) in the renal cortex of LPS-treated mice at 1 h. Levels of phospho-TPL-2 and phospho-ERK1/2 were normalized to their respective total protein content and actin was used as a secondary loading control. Data are presented as phosphorylated / total ratio relative to vehicle controls (mean ± S.E.M.; n = 4 / group for all analyses). Different superscripts above indicate statistically significant differences (p < 0.05) between groups.

Figure 6: Inhibition of MEK/ERK signaling prevents early changes in renal function and mitochondrial biogenesis in endotoxic AKI. Mice were pre-treated with the MEK1/2 inhibitor GSK1120212 (GSK112; 1 mg/kg) 1 h prior to systemic LPS exposure. Serum and kidneys were collected at 3 h post-LPS to determine the role of MEK/ERK signaling in early pathophysiology. Renal function was assessed by changes in BUN (**A**). The effects of MEK/ERK inhibition on tubular injury were determined by measuring KIM-1 mRNA expression in the renal cortex (**B**). PGC-1α transcript levels were determined by qPCR (**C**). Inhibition of ERK1/2 activation was confirmed by immunoblot analysis of phosphorylated and total ERK1/2 levels in the renal cortex (**D**). Data are shown as mean ± S.E.M.; n = 5 – 6 / group for all analyses; $p < 0.05$ vs. control).

Figure 7: Effects of GSK1120212 on LPS-induced AKI and suppression of mitochondrial biogenesis at 18 h. Mice receiving GSK1120212 (GSK112; 1 mg/kg) 1 h prior to endotoxin administration were euthanized at 18 h post-LPS for biochemical analysis. Renal dysfunction and tubular injury were determined by BUN (**A**) and KIM-1 mRNA expression (**B**), respectively. Transcript levels of PGC-1α (**C**) and NDUFS1 (**D**) were determined by qPCR analysis. Values are presented as mean ± S.E.M. for n ≥ 6 / group. Superscripts above bars are used to denote statistically significant differences compared to control ($p < 0.05$).

Figure 8: MEK/ERK signaling controls early renal expression of pro-inflammatory cytokines in response to endotoxin. Inflammatory changes were evaluated in the renal cortex of mice treated with LPS and GSK1120212 (GSK112; 1 mg/kg) as described above. qPCR was used to determine mRNA levels of TNF-α and IL-1β at both 3 h (**A**) and 18 h (**B**) post-LPS. Values are expressed as relative expression vs. control (mean ± S.E.M.) for n ≥ 6 / group ($p < 0.05$ vs control).

Figure 9: TNF-α mediates suppression of PGC-1α in the renal cortex of LPS-treated mice. Mice were treated with anti-TNF-α neutralizing antibody or the appropriate isotype control antibody 1 h prior to systemic LPS exposure and renal function and mitochondrial homeostasis were evaluated at 18 h. Renal dysfunction was determined by measuring BUN (**A**). Transcript levels of PGC-1α (**B**) and mitochondrial biogenesis markers (**C**) were assessed. qPCR analysis was used to determine relative mitochondrial DNA content (**D**). Data are mean ± S.E.M. (n = 4 / group; different superscripts indicate statistically significant differences ($p < 0.05$)).

Figure 10: Recombinant TNF-α induces AKI and renal mitochondrial dysfunction in mice. Mice were treated with recombinant TNF-α at variable doses (20 μg/kg or 50 μg/kg) or the appropriate diluent. Serum was collected at 18 h and BUN was determined (**A**). Total RNA and DNA were extracted from the renal cortex to assess expression of PGC-1α (**B**), downstream targets (**C**) and relative mitochondrial DNA content (**D**) by qPCR. Values are mean ± S.E.M. from n = 6 animals/group ($p < 0.05$ where denoted by different subscripts).

Figure 11: Proposed mechanisms of LPS-induced suppression of mitochondrial biogenesis in the renal cortex. Systemic LPS exposure leads to activation of TLR4 which in turn initiates signaling through the TPL-2/MEK/ERK pathway via phosphorylation (P). ERK1/2 activation contributes to multiple downstream effects of LPS including renal dysfunction, suppression of PGC-1α and subsequent mitochondrial biogenesis and production of pro-inflammatory cytokines such as TNF-α and IL-1β. Disruption of PGC-1α and MB leads to progressive renal dysfunction and prevents recovery after sepsis-induced AKI (Tran et al., 2011). TNF-α production also contributes to renal dysfunction and decreases PGC-1α transcription to further amplify LPS-mediated effects (Cunningham et al., 2002; Bhargava et al., 2013).

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Table 1: Primer pairs used for RT-qPCR.

Gene	Primer Sequence		Accession No.
PGC-1α	Sense	5'-AGGAAATCCGAGCGGAGCTGA-3'	NM_008904.2
	Antisense	5'-GCAAGAAGGCGACACATCGAA-3'	
PGC-1β	Sense	5'-CTCTGACGCTCTGAAGGACG-3'	NM_133249.2
	Antisense	5'-GTGCCATCCACCTTGACACA-3'	
PRC	Sense	5'-ATTCAGAGCTGCTCGTGTCC-3'	NM_001081214.1
	Antisense	5'-GGGCCCCAAAGGGTCAAT-3'	
NRF1	Sense	5'-TCGGGCATTTATCCCAGAGATGCT-3'	NM_001164226.1
	Antisense	5'-TACGAGATGAGCTATACTGTGTGT-3'	
TFAM	Sense	5'-GCTGATGGGTATGGAGAAG-3'	NM_009360.4
	Antisense	5'-GAGCCGAATCATCCTTTGC-3'	
NDUFS1	Sense	5'-AGATGATTTGGGAACAACAG-3'	NM_001160038.1
	Antisense	5'-TAAGGCTTAGAGGTTAGGGC-3'	
NDUFB8	Sense	5'-CGCCAAGAAGTATAACATGC-3'	NM_026061.2
	Antisense	5'-TCATCCTGAGTTCTGAGTGG-3'	
ATPSβ	Sense	5'-CTATGTGCCTGCTGATGACC-3'	NM_016774.3
	Antisense	5'-GGATAGATGCCCAACTCAGC-3'	
COXI	Sense	5'-ACCATCATTTCTCCTTCTCC-3'	ENSMUSG00000064351.1
	Antisense	5'-GGTGGGTAGACTGTTCATCC-3'	
ND1	Sense	5'- TAGAACGCAAAATCTTAGGG-3'	ENSMUSG00000064341.1
	Antisense	5'- TGCTAGTGTGAGTGATAGGG-3'	
TNF-α	Sense	5'-TCCCTCTCATCAGTTCTATGGCCCA-3'	NM_013693.3
	Antisense	5'- GTGTGGGTGAGGAGCACGTA-3'	
IL-1β	Sense	5'- TGCCACCTTTTGACAGTGATG-3'	NM_008361.3
	Antisense	5'-ATGTGCTGCTGCGAGATTTG-3'	

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ICAM-1	Sense	5’-CACGTGCTGTATGGTCCTCG-3’	NM_010493.2
	Antisense	5’-TAGGAGATGGGTTCCCCCAG-3’	
E-Selectin	Sense	5’-TTCGGCACAGTGTGTGAGTT-3’	NM_011345.2
	Antisense	5’-TGACTGGGGCTTCACAGGTA-3’	
KIM-1	Sense	5’-GCATCTCTAAGCGTGGTTGC-3’	NM_134248.2
	Antisense	5’-TCAGCTCGGGAATGCACAA-3’	
NGAL	Sense	5’-ATGCACAGGTATCCTCAGGT-3’	NM_008491.1
	Antisense	5’-ACCATGGCGAACTGGTTGT-3’	
18S rRNA	Sense	5’-TTGACGGAAGGGCACCACCAG-3’	NR_003278.3
	Antisense	5’-GCACCACCACCCACGGAATCG-3’	

Figure 1

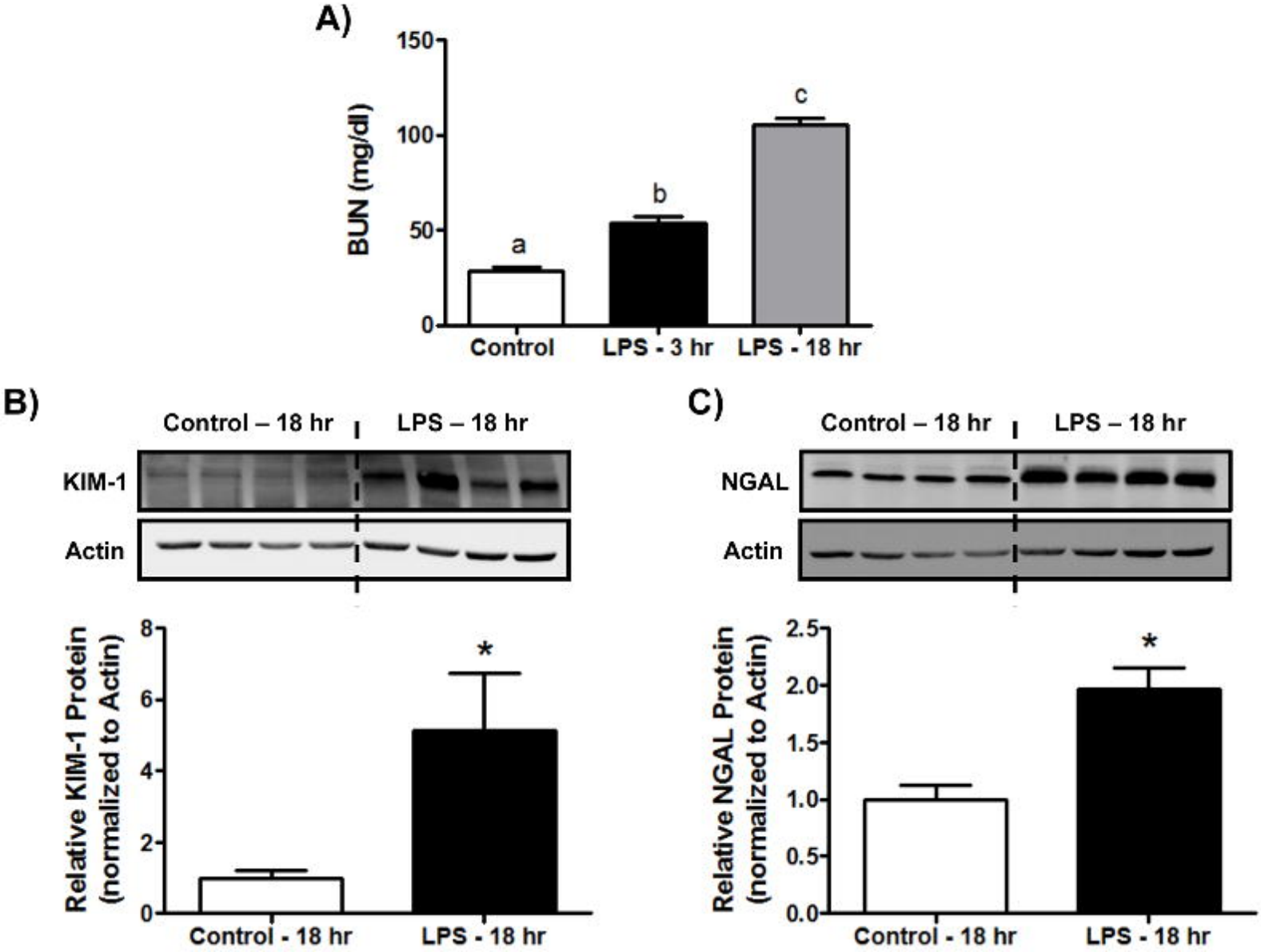

A)
BUN (mg/dl)
150
100
50
0
a
b
c
Control
LPS - 3 hr
LPS - 18 hr
B)
Control – 18 hr
LPS – 18 hr
KIM-1
Actin
Relative KIM-1 Protein (normalized to Actin)
8
6
4
2
0
*
Control - 18 hr
LPS - 18 hr
C)
Control – 18 hr
LPS – 18 hr
NGAL
Actin
Relative NGAL Protein (normalized to Actin)
2.5
2.0
1.5
1.0
0.5
0.0
*
Control - 18 hr
LPS - 18 hr

Figure 2

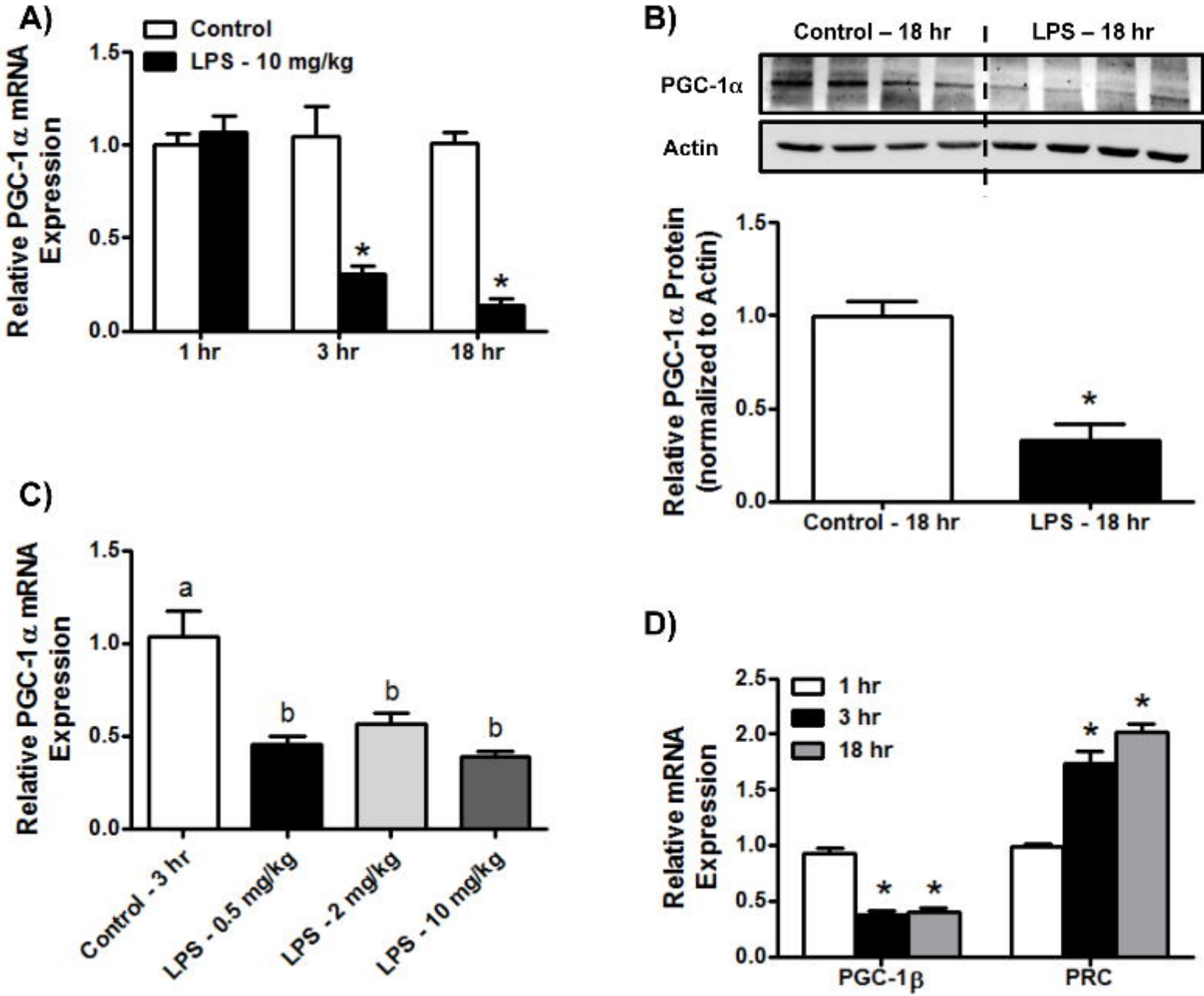

A)
Control
LPS - 10 mg/kg
Relative PGC-1α mRNA Expression
1.5
1.0
0.5
0.0
*
*
1 hr
3 hr
18 hr
B)
Control – 18 hr
LPS – 18 hr
PGC-1α
Actin
Relative PGC-1α Protein (normalized to Actin)
1.5
1.0
0.5
0.0
*
Control - 18 hr
LPS - 18 hr
C)
Relative PGC-1α mRNA Expression
1.5
1.0
0.5
0.0
a
b
b
b
Control - 3 hr
LPS - 0.5 mg/kg
LPS - 2 mg/kg
LPS - 10 mg/kg
D)
1 hr
3 hr
18 hr
Relative mRNA Expression
2.5
2.0
1.5
1.0
0.5
0.0
*
*
*
*
PGC-1β
PRC

Figure 3

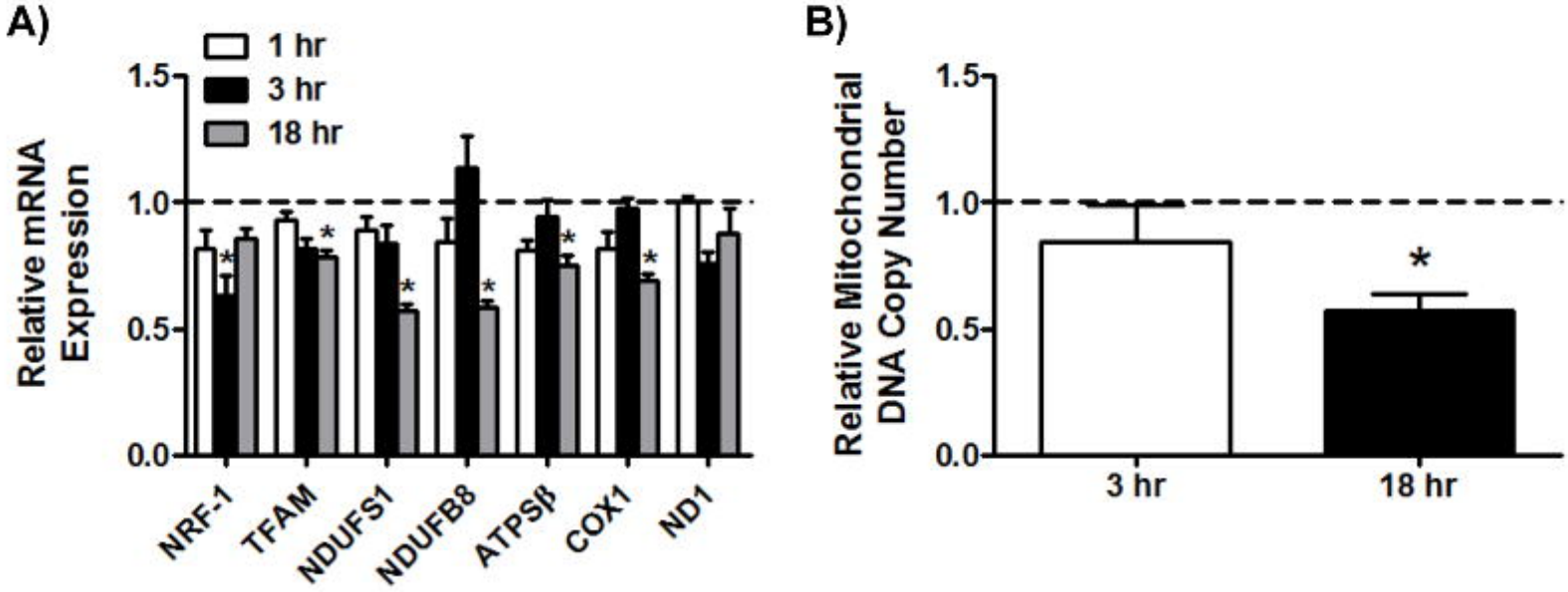

A)
1 hr
3 hr
18 hr
Relative mRNA Expression
1.5
1.0
0.5
0.0
NRF-1
TFAM
NDUFS1
NDUFB8
ATPSβ
COX1
ND1
B)
Relative Mitochondrial DNA Copy Number
1.5
1.0
0.5
0.0
3 hr
18 hr

Figure 4

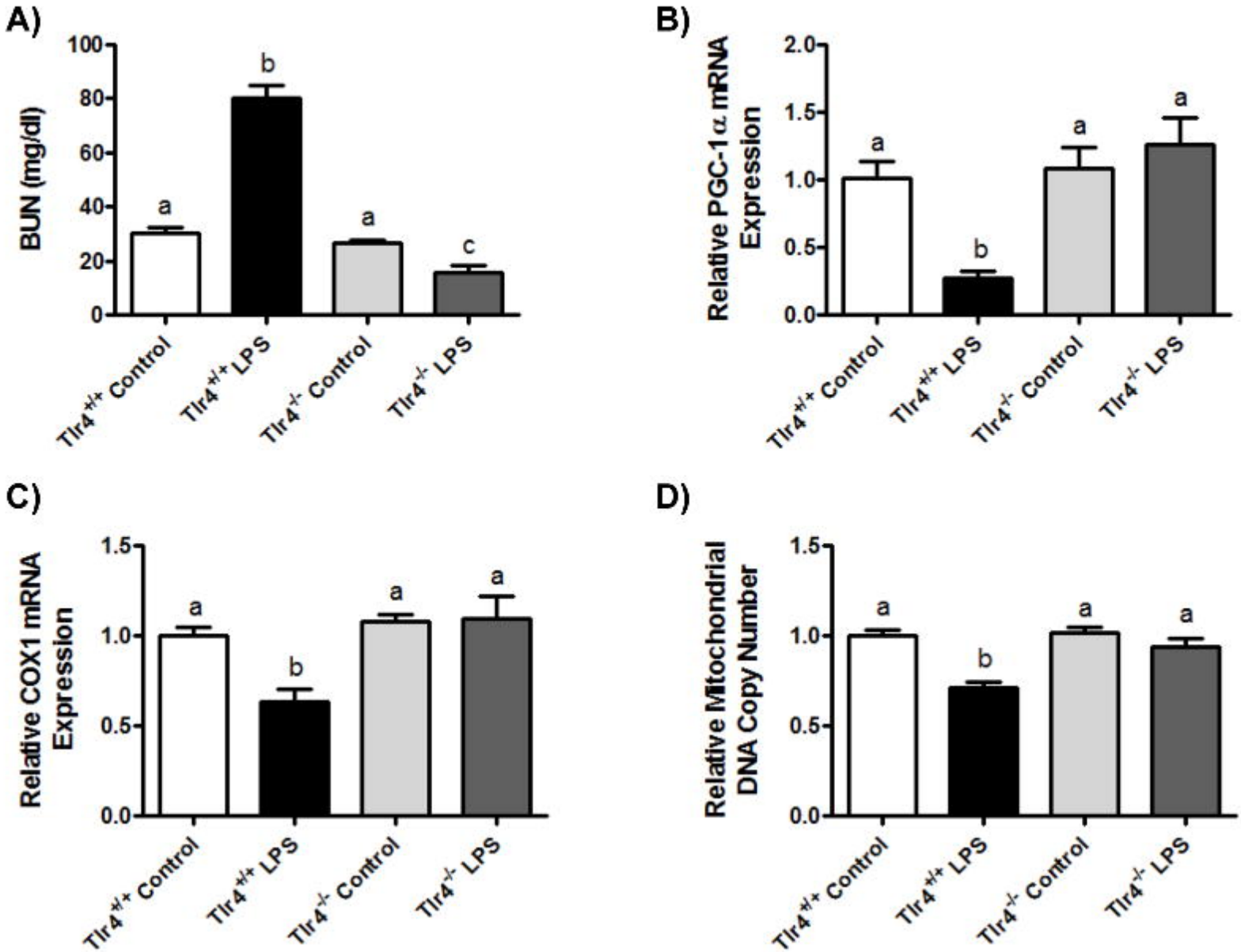

A)
BUN (mg/dl)
100
80
60
40
20
0
a
b
a
c
Tlr4+/+ Control
Tlr4+/+ LPS
Tlr4-/- Control
Tlr4-/- LPS
B)
Relative PGC-1α mRNA Expression
2.0
1.5
1.0
0.5
0.0
a
b
a
a
Tlr4+/+ Control
Tlr4+/+ LPS
Tlr4-/- Control
Tlr4-/- LPS
C)
Relative COX1 mRNA Expression
1.5
1.0
0.5
0.0
a
b
a
a
Tlr4+/+ Control
Tlr4+/+ LPS
Tlr4-/- Control
Tlr4-/- LPS
D)
Relative Mitochondrial DNA Copy Number
1.5
1.0
0.5
0.0
a
b
a
a
Tlr4+/+ Control
Tlr4+/+ LPS
Tlr4-/- Control
Tlr4-/- LPS

Figure 5

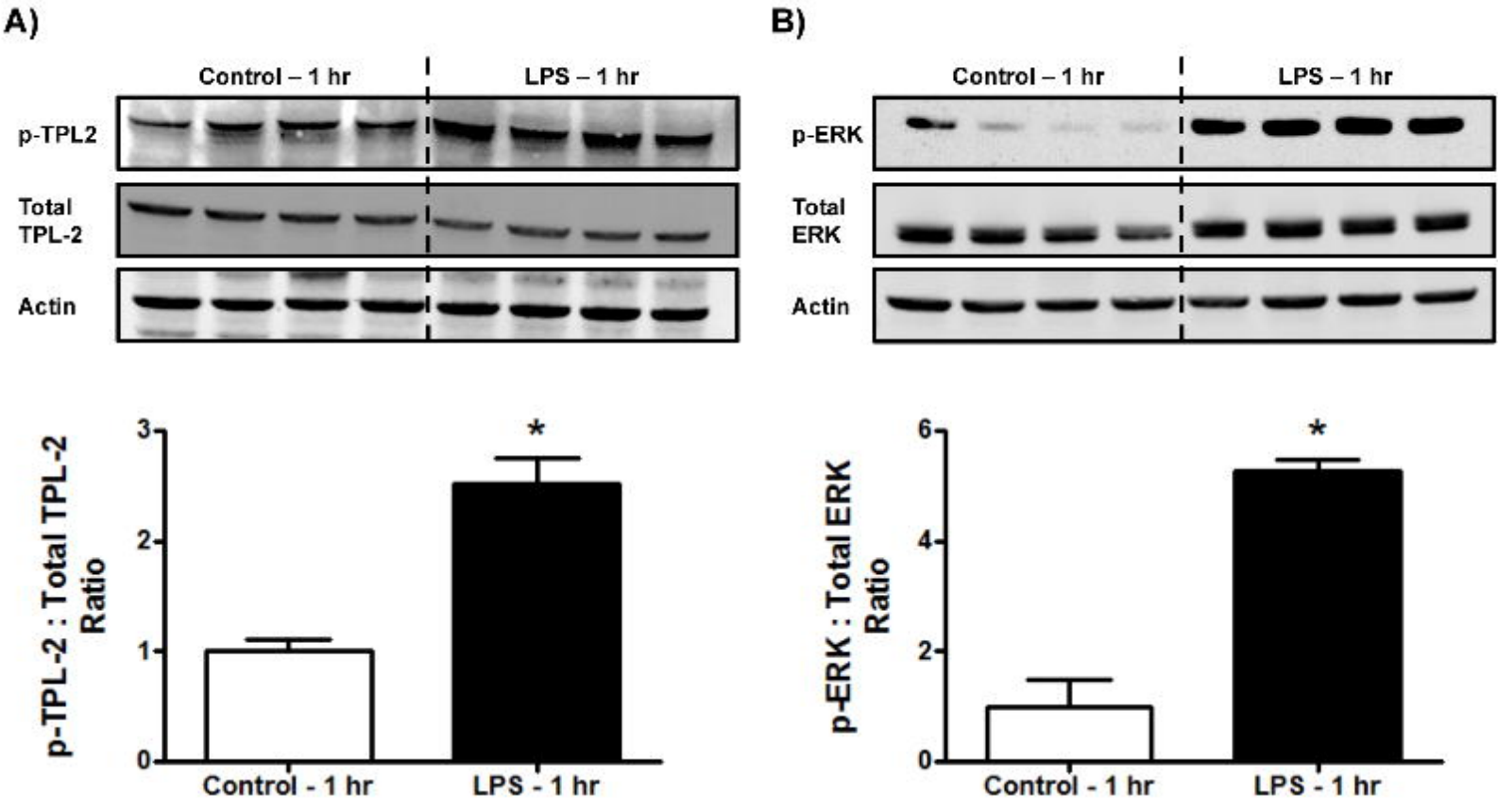

A)
Control – 1 hr
LPS – 1 hr
p-TPL2
Total TPL-2
Actin
B)
Control – 1 hr
LPS – 1 hr
p-ERK
Total ERK
Actin
p-TPL-2 : Total TPL-2 Ratio
0
1
2
3
*
Control - 1 hr
LPS - 1 hr
p-ERK : Total ERK Ratio
0
2
4
6
*
Control - 1 hr
LPS - 1 hr

Figure 6

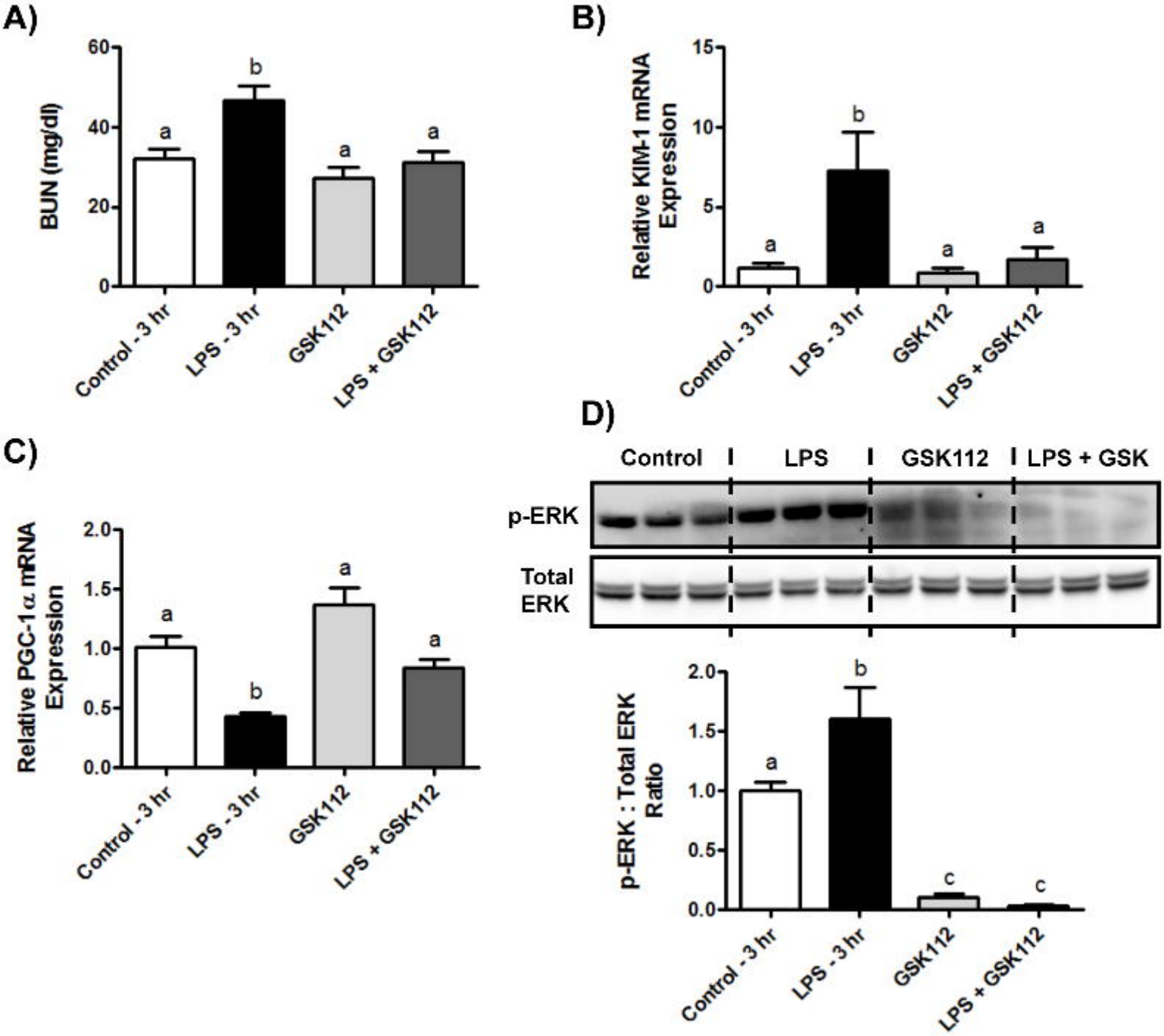

A)
BUN (mg/dl)
60
40
20
0
a
b
a
a
Control - 3 hr
LPS - 3 hr
GSK112
LPS + GSK112
B)
Relative KIM-1 mRNA Expression
15
10
5
0
a
b
a
a
Control - 3 hr
LPS - 3 hr
GSK112
LPS + GSK112
C)
Relative PGC-1α mRNA Expression
2.0
1.5
1.0
0.5
0.0
a
b
a
a
Control - 3 hr
LPS - 3 hr
GSK112
LPS + GSK112
D)
Control
LPS
GSK112
LPS + GSK
p-ERK
Total ERK
p-ERK : Total ERK Ratio
2.0
1.5
1.0
0.5
0.0
a
b
c
c
Control - 3 hr
LPS - 3 hr
GSK112
LPS + GSK112

Figure 7

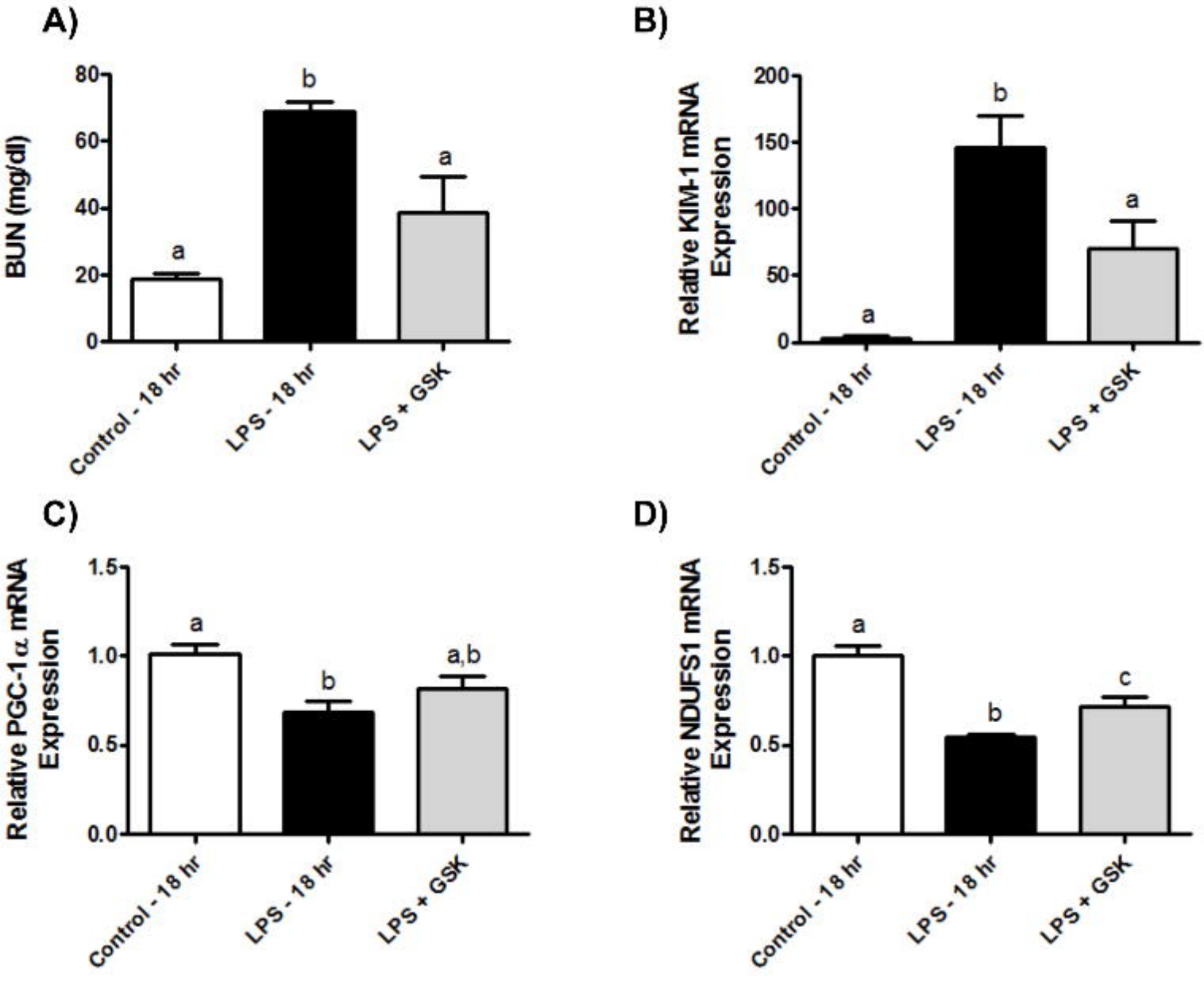
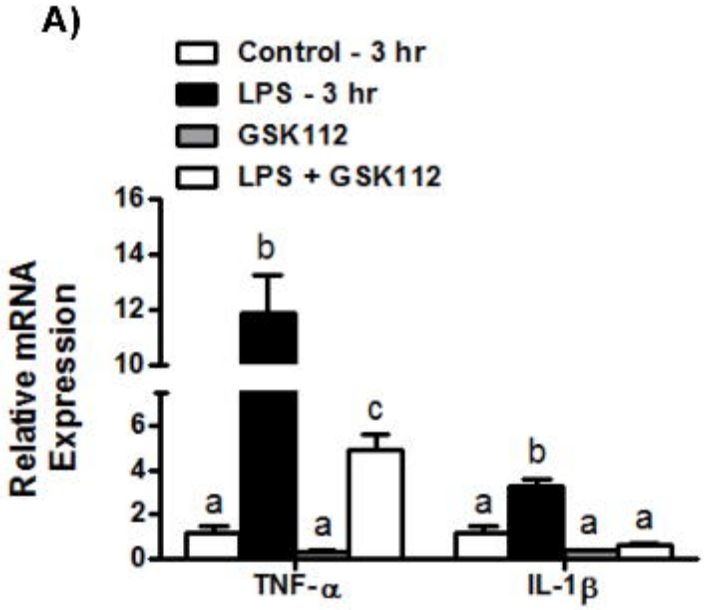

A)
BUN (mg/dl)
80
60
40
20
0
a
b
a
Control - 18 hr
LPS - 18 hr
LPS + GSK
B)
Relative KIM-1 mRNA Expression
200
150
100
50
0
a
b
a
Control - 18 hr
LPS - 18 hr
LPS + GSK
C)
Relative PGC-1α mRNA Expression
1.5
1.0
0.5
0.0
a
b
a,b
Control - 18 hr
LPS - 18 hr
LPS + GSK
D)
Relative NDUFS1 mRNA Expression
1.5
1.0
0.5
0.0
a
b
c
Control - 18 hr
LPS - 18 hr
LPS + GSK

Figure 8


A)
Control - 3 hr
LPS - 3 hr
GSK112
LPS + GSK112
Relative mRNA Expression
16
14
12
10
6
4
2
0
a
b
a
c
a
b
a
a
TNF-α
IL-1β

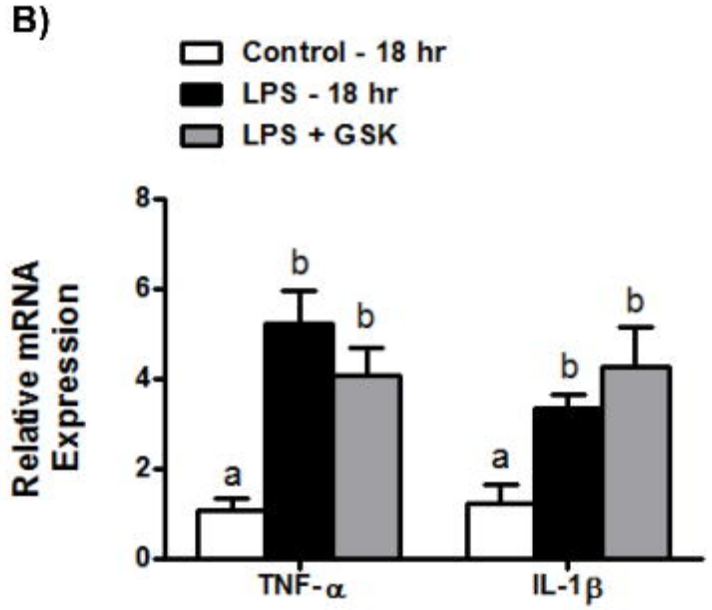

B)
Control - 18 hr
LPS - 18 hr
LPS + GSK
Relative mRNA Expression
8
6
4
2
0
a
b
b
a
b
b
TNF-α
IL-1β

Figure 9

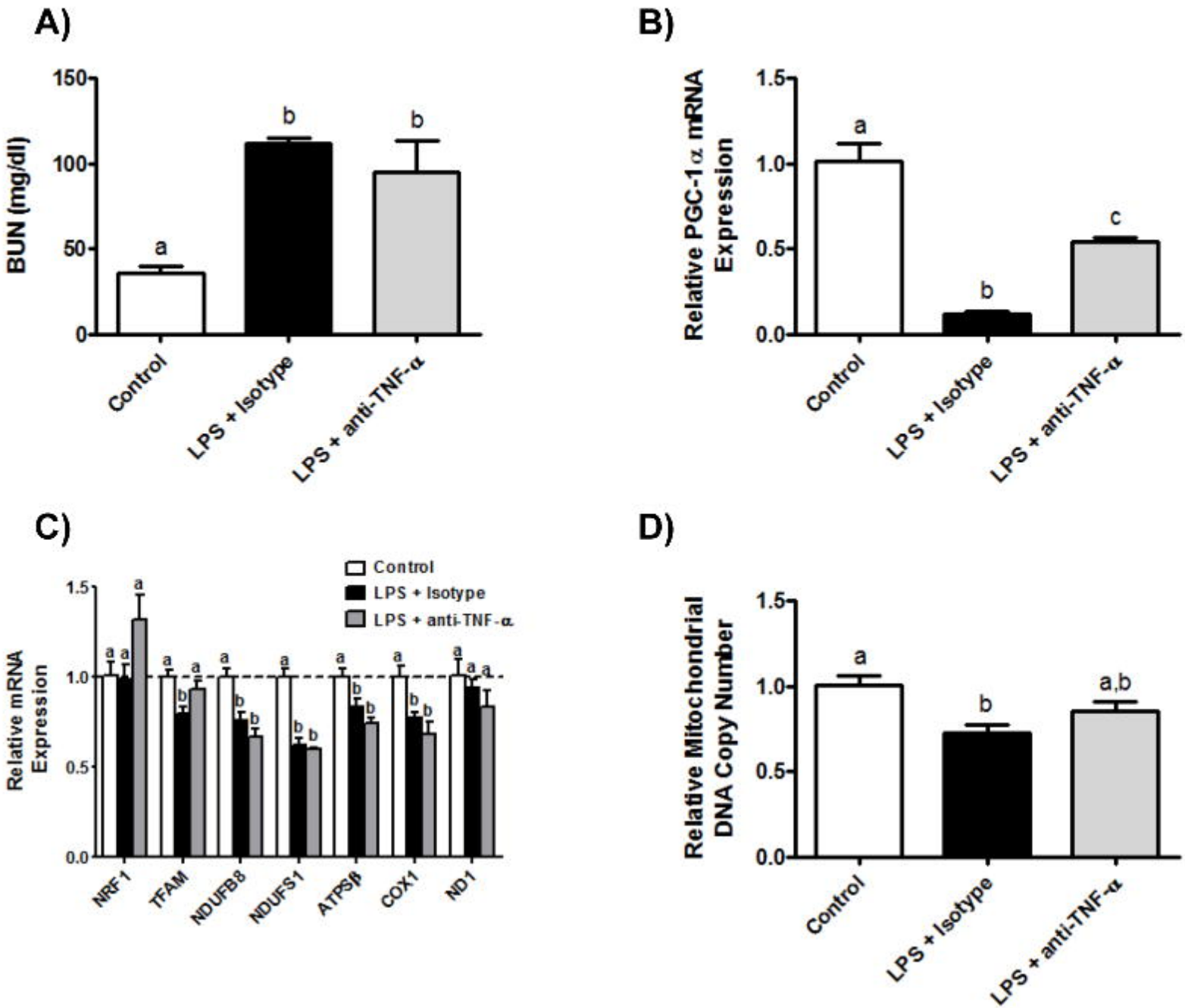

A)
BUN (mg/dl)
150
100
50
0
a
b
b
Control
LPS + Isotype
LPS + anti-TNF-α
B)
Relative PGC-1α mRNA Expression
1.5
1.0
0.5
0.0
a
b
c
Control
LPS + Isotype
LPS + anti-TNF-α
C)
Control
LPS + Isotype
LPS + anti-TNF-α
Relative mRNA Expression
1.5
1.0
0.5
0.0
NRF-1
TFAM
NDUFB8
NDUFS1
ATPSβ
COX1
ND1
D)
Relative Mitochondrial DNA Copy Number
1.5
1.0
0.5
0.0
a
b
a,b
Control
LPS + Isotype
LPS + anti-TNF-α

Figure 10

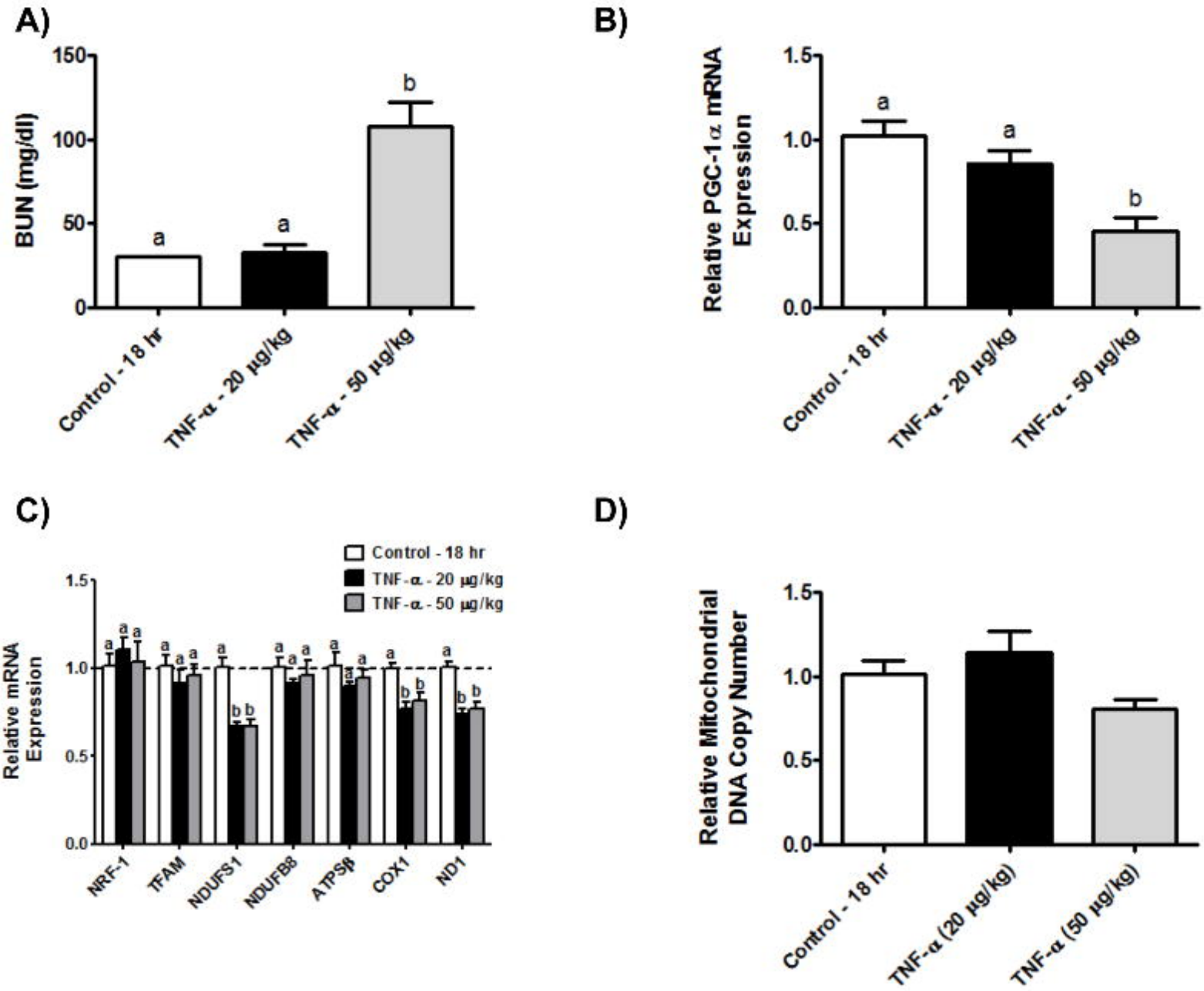
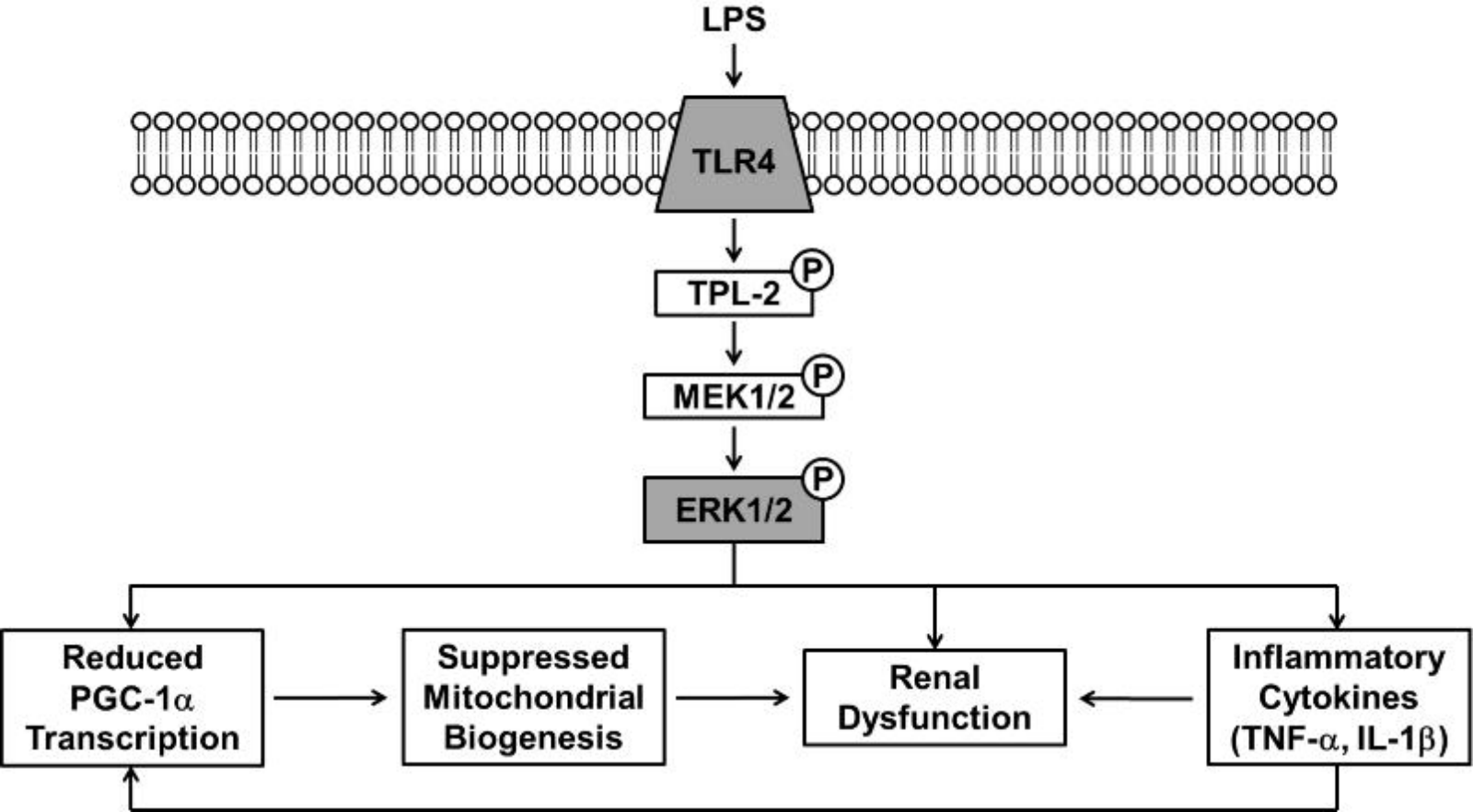

A)
BUN (mg/dl)
150
100
50
0
a
a
b
Control - 18 hr
TNF-α - 20 µg/kg
TNF-α - 50 µg/kg
B)
Relative PGC-1α mRNA Expression
1.5
1.0
0.5
0.0
a
a
b
Control - 18 hr
TNF-α - 20 µg/kg
TNF-α - 50 µg/kg
C)
Control - 18 hr
TNF-α - 20 µg/kg
TNF-α - 50 µg/kg
Relative mRNA Expression
1.5
1.0
0.5
0.0
NRF-1
TFAM
NDUFS1
NDUFB8
ATPSβ
COX1
ND1
D)
Relative Mitochondrial DNA Copy Number
1.5
1.0
0.5
0.0
Control - 18 hr
TNF-α (20 µg/kg)
TNF-α (50 µg/kg)

Figure 11


LPS
TLR4
TPL-2
P
MEK1/2
P
ERK1/2
P
Reduced PGC-1α Transcription
Suppressed Mitochondrial Biogenesis
Renal Dysfunction
Inflammatory Cytokines (TNF-α, IL-1β)