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**TITLE PAGE**

# SRT1720 Induces Mitochondrial Biogenesis and Rescues Mitochondrial Function After Oxidant Injury in Renal Proximal Tubule Cells

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**Running title:** SRT1720 induces mitochondrial biogenesis

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**List of non-standard abbreviations:** Aminoimidazole carboxamide ribonucleotide (AICAR), Adenosine monophosphate (AMP)-activated kinase (AMPK), nicotinamide (NAM), NADH-ubiquinone oxidoreductase chain 6 (ND6), NADH dehydrogenase 1 beta

subcomplex subunit 8 (NDUFB8), peroxisome proliferator activated receptor coactivator-1 alpha (PGC-1α), renal proximal tubule cells (RPTC), mammalian sirtuin 1 (SIRT1), *tert*butyl hydroperoxide (TBHP)

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## ABSTRACT

Mitochondrial biogenesis occurs under basal conditions and is an adaptive response initiated by cells to maintain energetic demands and metabolic homeostasis following injuries targeting mitochondrial function. Identifying pharmacological agents that stimulate mitochondrial biogenesis is a critical step in the development of new therapeutics for the treatment of these injuries and to test the hypothesis that these agents will expedite recovery of cell and organ function following acute organ injuries. In this study, we examined the effects of SRT1720 on mitochondrial biogenesis and function in primary cultures of renal proximal tubule cells (RPTC). We also tested the ability of this compound to restore mitochondrial functions following oxidant-induced RPTC injury. SRT1720 (3-10 μM) induced mitochondrial biogenesis in RPTC within 24 hrs as determined by elevations in mitochondrial DNA copy number, increased expression of the mitochondrial proteins NDUFB8 and ATP synthase β, and elevated mitochondrial respiration rates and ATP levels. Induction of mitochondrial biogenesis was dependent on SIRT1 deacetylase activity, correlated with deacetylated nuclear PGC-1α, and occurred in the absence of AMP-dependent kinase (AMPK) activation. Finally, SRT1720 treatment accelerated recovery of mitochondrial functions following acute oxidant injury. This study demonstrates that SRT1720 can induce mitochondrial biogenesis through SIRT1 activity and deacetylated PGC-1α, but not AMPK, in RPTC within 24 hrs following oxidant injury. The results support further study of mitochondrial biogenesis as a repair process and a pharmacological target in acute organ injuries and disorders plagued by mitochondrial impairment.

## INTRODUCTION

Mitochondrial dysfunction is a primary pathological consequence of ischemic or toxic insults. In ischemic acute kidney injury (AKI), de-energization of the mitochondria and persistent energy depletion may hinder critical energy-dependent repair mechanisms and lead to irreversible cell injury, limiting restoration of organ function (Weinberg et al., 2000; Feldkamp et al., 2005). As such, there is therapeutic potential for agents that promote mitochondrial function to treat injuries characterized by mitochondrial impairment.

Mitochondrial biogenesis occurs under basal conditions and is an adaptive response initiated by cells to maintain energy demands or heat expenditure following injury, cold exposure, or caloric restriction (Puigserver et al., 1998; Yin et al., 2008). A primary regulator of mitochondrial biogenesis is the nuclear transcriptional coactivator peroxisome proliferator-activated receptor coactivator-1α (PGC-1α). Through induction of uncoupling proteins (UCP-2), nuclear respiratory factors (NRF1/2), and as a coactivator of the promoter region of mitochondrial transcription factors Tfam, TFB1M, and TFB2M, PGC-1α has significant influence on mitochondrial function (Puigserver et al., 1998; Wu et al., 1999; Puigserver and Spiegelman, 2003; Gleyzer et al., 2005; Lin et al., 2005).

PGC-1α is highly expressed in metabolic tissues, and its expression and activity are regulated by a network of receptors, including the nuclear hormone receptors thyroid

hormone and PPARγ (Puigserver et al., 1998; Wrutniak-Cabello et al., 2001); signaling pathways, such as the MAPK and CaMK pathways (Barger et al., 2001; Wu et al., 2002); and post-translational phosphorylation, methylation, and acetylation modifications (Barger et al., 2001; Puigserver et al., 2001; Teyssier et al., 2005; Coste et al., 2008). Additionally, PGC-1α transcription is regulated by the activity of signaling molecules and transcription factors such as protein kinase B, forkhead transcription factor, and myocyte enhancer factor-2 (MEF-2) (Czubryt et al., 2003; Daitoku et al., 2003).

Under energetic crises, primary responders for maintaining energy and nutrient homeostasis are AMP-activated kinase (AMPK) and SIRT1. Rather than exclusive mechanisms of adaptation, recent evidence points to concurrent regulation and convergent mechanisms induced by AMPK and SIRT1 in response to changes in cellular energy levels and redox states, with a primary target of both pathways converging on PGC-1α (Canto et al., 2009). AMPK monitors cellular energy levels, inducing ATP synthesis and inhibiting ATP expenditure when ATP levels are low (Hardie et al., 2003), by regulating expression of mitochondrial and metabolic genes via direct phosphorylation of PGC-1α (Jager et al., 2007). SIRT1 is a nuclear protein that is also activated in response to energy depletion, and promotes induction of genes that regulate metabolic adaptation to low energy levels. As a member of a conserved family of $NAD^+$-dependent deacetylase enzymes known as the *sirtuins,* SIRT1 monitors cellular energy levels and becomes active in response to elevated $NAD^+$/NADH ratios (Landry et al., 2000). SIRT1 catalyzes the deacetylation and activation of PGC-1α in both *in vitro* and *in vivo* systems (Nemoto et al., 2005; Rodgers et al., 2005), which may contribute to a protective role in

metabolic regulation and resistance to oxidative stress (Howitz et al., 2003; Bordone and Guarente, 2005)

A number of small molecules have been reported, such as resveratrol and isoflavone-derived compounds (Howitz et al., 2003; Rasbach and Schnellmann, 2008), to induce mitochondrial biogenesis in RPTC. SRT1720 was reported to be a SIRT1 activator, and exposure of this compound led to deacetylation of SIRT1 target proteins in both cells and animals (Milne et al., 2007; Feige et al., 2008). In genetic and diet-induced obese and diabetic rodents, 4- to 10-weeks of SRT1720 treatment improves insulin sensitivity and reduces plasma glucose levels while enhancing skeletal muscle mitochondrial activity (Milne et al., 2007). Additionally, C2C12 cells treated with SRT1720 express elevated citrate synthase activity and ATP levels, suggesting induction of mitochondrial biogenesis (Smith et al., 2009). However, the acute effects of this compound in primary cultures of renal proximal tubule cells, which better mimic the metabolic properties of cells *in vivo* compared to glycolytic cell lines, on mitochondrial biogenesis have not been explored. Furthermore, the effects of this compound in targeted injury models with mitochondrial impairment have also not been characterized.

Mitochondrial dysfunction contributes to oxidant-induced renal cell injury (Nowak et al., 1998), and PGC-1α plays a predominant role in the recovery of mitochondrial function following the initial injury (Rasbach and Schnellmann, 2007b). Over-expression of PGC-1α accelerates recovery of mitochondrial and cellular functions after oxidant injury (Rasbach and Schnellmann, 2007a), but because of the toxicity limitations in using

adenovirus in vivo, there is a need for pharmacological agents that stimulate mitochondrial biogenesis to treat injuries characterized by mitochondrial impairment. In this study, we examined the mechanism of SRT1720 induced mitochondrial biogenesis and function in renal epithelial cells and tested the hypothesis that stimulation of mitochondrial function accelerates recovery from an acute cellular and mitochondrial injury.

## METHODS

*Reagents* – PGC-1α (H300), NDUFB8, ATP Synthase β, and GAPDH antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), Invitrogen (Carlsbad, CA), Abcam (Cambridge, MA), and Fitzgerald (Concord, MA), respectively. Acetylated lysine, phosphorylated AMPK (Thr172), and AMPK antibodies were obtained from Cell Signaling Technologies (Danvers, MA). Sirtinol and nicotinamide were purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals were obtained from Sigma-Aldrich.

*SRT1720 synthesis and SIRT1 activation* – SRT1720 was synthesized according to a procedure previously described (Milne et al., 2007) and was confirmed by NMR and mass spectrometry and the final product was purified by HPLC. SIRT1 deacetylase activity was measured using a fluorescence-based SIRT1 activity kit (BioMol, Plymouth Meeting, PA) according to manufacturer's protocol as previously described (Rasbach and Schnellmann, 2008).

*Isolation and Culture of Renal Proximal Tubules* – Female New Zealand White rabbits (~2 kg) were purchased from Myrtle's Rabbitry (Thompson Station, TN). Renal proximal

tubules were isolated using an iron oxide uptake method previously described (Rasbach and Schnellmann, 2007b). Cells were cultured on 35 mm dishes in a medium consisting of 1:1 DMEM/Ham's F12 (lacking glucose, phenol red, and sodium pyruvate), and supplemented with HEPES (15 mM), glutamine (2.5 mM), pyridoxine HCl (1 uM), sodium bicarbonate (15 mM), and lactate (6 mM). Hydrocortisone (50 nM), selenium (5 ng/ml), human transferrin (5 µg/ml), bovine insulin (10 nM), and L-ascorbic acid-2-phosphate (50 µM) were added daily to fresh culture medium. Experiments with RPTC were conducted on the sixth day after plating when the cells had reached a confluent monolayer. Treatments were administered for 24 hrs unless otherwise noted. For TBHP injury experiments, cells were exposed to 400 µM TBHP for 45 min, at which time TBHP media was replaced with fresh media.

*Preparation of cell lysates for immunoblot analysis* – Twenty-four hours following treatment, RPTCs were harvested in RIPA lysis buffer consisting of 25 mM Tris-HCl, 150 mM NaCl, 1% TritonX-100, 1% sodium deoxycholate, and 0.1% SDS. Lysates were sonicated and total protein was measured by BCA. Immunoblot analysis was performed as previously described (Rasbach and Schnellmann, 2007b).

*Immunoprecipitation* – Cells were harvested from pooled culture dishes in a homogenization buffer consisting of 50 mM Tris-HCl, 1 mM β-mercaptoethanol, 1 mM EDTA, and 0.32 M sucrose. Cells were disrupted by sonication and nuclei were collected by centrifugation at 900 x g for 10 min. Following centrifugation, the nuclear pellet was resuspended in a nuclear lysis buffer consisting of 10 mM Tris, 500 mM NaCl, 1% TritonX-100, 10% glycerol, 1 mM sodium pyrophosphate, 1 mM $NaVO_4$, 1 mM NaF,

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and protease inhibitors. Immunoprecipitations were carried out according to a protocol by Roche Diagnostics (Indianapolis, IN). Nuclear protein lysate (500 µg) and 5 µg PGC-1α antibody were used for experiments. Immunoprecipitates were analyzed by immunoblotting using antibodies against acetylated lysine residues and PGC-1α. Supernatants collected from immunoprecipitations were analyzed for Histone H3 expression as a control for initial nuclear protein input.

*Quantitative Real-Time PCR* – Total RNA was isolated from cells with TRIzol (Invitrogen, Carlsbad, CA). cDNA was synthesized from 5 µg RNA template using a SuperScript II Reverse Transcriptase kit (Invitrogen). PCR reactions were carried out using 2.5 µL cDNA template combined with Brilliant II SYBR Green master mix (Stratagene) at a final concentration of 1X and primers (Integrated DNA Technologies) at a concentration of 400 nM. Sequences of primers used for real-time PCR reactions: PGC-1α (FW: 5'-AGG AAA TCC GAG CTG AGC TGA ACA-3', REV: 5'-GCA AGA AGG CGA CAC ATC GAA CAA-3'), and GAPDH (FW: 5'-GAG CTG AAC GGG AAA CTC AC-3', REV: 5'-CAC TGT TGA AGT CGC AGG AG-3').

*Mitochondrial DNA content* – Real-time PCR was used to determine relative quantities of mitochondrial DNA content in SRT1720-treated cells and control cells. Following a 24 hr treatment, total genomic DNA was extracted using a DNeasy Blood and Tissue Kit (Qiagen). DNA was quantified by measuring A260 values and 50 ng total DNA was used for PCR reactions. Primers specific to the mitochondrial-encoded ND6 gene (FW: ACT GCG ATG GCA ACT GAG GAG TAT, REV: ACC ATA ACT ATA CAA CGC CGC CAC) were used to assess mitochondrial DNA copy numbers. Primers designed

against the nuclear-encoded Pou5f1 gene (FW: 5'-GGC CTA TGT CTT TTC CTC TGG-3', REV: 5'-TCC AGG TTC TCT CTC CCT AGC-3') were used for normalization.

*Oxygen Consumption ($QO_2$) and ATP levels* – Basal and FCCP-uncoupled oxygen consumption ($QO_2$) and ATP levels were measured 24 hr following treatment with SRT1720 and/or TBHP. $QO_2$ was measured using a Clark oxygen electrode as previously described (Nowak et al., 1998). ATP content was measured using an ATP bioluminescent assay kit (BioMol) as previously described and normalized to cellular protein (Rasbach and Schnellmann, 2007b).

*Statistical Analysis*—Data are presented as means +/- SEM and were subjected to one-way ANOVA. Multiple means were compared post-hoc using Student-Newman-Keuls test were considered statistically different when $p<0.05$. RPTC isolated from a single rabbit represented an individual experiment ($n$=1) and were repeated until an $n$ of at least 6 was obtained.

**RESULTS**

SRT1720 was reported to activate SIRT1 (Milne et al., 2007; Feige et al., 2008), and because SIRT1 activation can increase PGC-1α activity and mitochondrial functioning, we conducted a series of experiments to determine if SRT1720 mediates mitochondrial biogenesis in primary cultures of RPTC, and if so, by what mechanism. To verify SRT1720 potency, a fluorescence-based SIRT1 activity assay kit measuring deacetylation of a peptide target was used to examine SIRT1 deacetylase activity when exposed to

SRT1720. SRT1720 increased SIRT1 activity in a concentration-dependent manner with a 3-fold increase in SIRT1 at 1 and 3 μM SRT1720 and a 5-fold increase at 10 μM (Fig. 1).

Because SIRT1 can modulate PGC-1α expression and/or activity by deacetylation (Nemoto et al., 2005; Rodgers et al., 2005), we assessed the expression and acetylation state of PGC-1α in RPTC exposed to SRT1720 or vehicle for 24 hrs. Immunoblot analysis of nuclear lysates revealed elevated PGC-1α expression (Fig. 2a). To further examine nuclear PGC-1α content and acetylation state, PGC-1α protein was immunoprecipitated from nuclear lysates and subjected to immunoblot analysis with antibodies to acetylated lysine residues and PGC-1α (Fig. 2b). Time course analysis of acetylated PGC-1α consistently revealed reduced acetylation with 48 hr SRT1720 treatment with no differences at 24 hrs. Total PGC-1α levels in the immunoprecipitate were elevated at 24 and 48 hrs. The ratio of acetylated to total PGC-1α was decreased approximately 50% in SRT1720 cells at 24 hrs, indicating more active PGC-1α in the nucleus with SRT1720 treatment. We confirmed equal loading by measuring histone H3 in the supernatant from the immunoprecipitation experiments by immunoblot analysis (Fig. 2b).

Because active PGC-1α promotes transcription of the PGC-1 gene by an autoregulatory feedback loop (Czubryt et al., 2003), we examined transcript levels of PGC-1α by real-time PCR, but found no differences between vehicle and SRT1720-treated cells (Fig. 2c). Because modifications to PGC-1α may regulate degradation of the protein, we tested

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whether the increased expression of PGC-1α was due to decreased proteasomal degradation. We have previously characterized the degradation of PGC-1α in RPTC, and showed that it has a short half-life (37 min) (Rasbach et al., 2008). RPTC were treated with vehicle or SRT1720 for 24 hrs and then protein translation was inhibited with cycloheximide (100 μM) and samples taken 30 and 60 min later. Nuclear lysates were probed for PGC-1α expression by immunoblot analysis. No changes in PGC-1α degradation were observed SRT1720 and vehicle treated RPTC (Fig.2d). Taken together, these data provide evidence that SRT1720 treatment induced accumulation of deacetylated nuclear PGC-1α in RPTC that was not the result of either elevated PGC-1α transcription at 24 hrs treatment or decreased proteasomal degradation.

Mitochondrial biogenesis was determined by assessing mitochondrial DNA copy number, expression of mitochondrial proteins, and mitochondrial function after 24 hrs of SRT1720 treatment. Relative mitochondrial DNA copy number was determined using quantitative real-time PCR to examine the ratio of a select mitochondrial-encoded gene over nuclear DNA in SRT1720 and vehicle-treated cells (Fig. 3a). There was a 3.5-fold increase in mitochondrial-encoded NADH dehydrogenase subunit 6 (ND6) DNA in SRT1720 cells compared to controls. Nuclear-encoded Pou5f1 was used for normalization.

Secondly, the effect of SRT1720 treatment on mitochondrial protein levels was explored. SRT1720 (10 μM) elevated ATP Synthase β, a nuclear-encoded protein within the $F_1$ subunit of the ATP synthase, 1.5-fold over controls (Fig. 3b). NDUFB8, a nuclear-

encoded complex I subunit, also was elevated approximately 1.5-fold over control by SRT1720.

Mitochondrial function was determined by measuring cellular respiration and ATP levels in RPTC. Compared to controls, basal respiration was elevated approximately 1.5-fold with 3 or 10 μM treatments at 24 hrs (Fig. 3c). Uncoupled respiration was elevated approximately 1.5-fold at the same concentrations. Finally, ATP levels were also elevated (1.8-fold) over vehicle controls (Fig. 3c). Taken together, the elevations in mitochondrial DNA, proteins, and functional capacity provide strong evidence that mitochondrial biogenesis occurs in RPTC with SRT1720 treatment.

To verify that the RPTC mitochondrial biogenesis produced by SRT1720 is dependent on SIRT1 activation, pharmacologic inhibitors were used to block SIRT1 activity prior to SRT1720 exposure, and then mitochondrial DNA content and function were analyzed. SRT1720 treatment elevated mitochondrial DNA content compared to vehicle treated cells, whereas cells exposed to SRT1720 in the presence of the SIRT1 inhibitor nicotinamide (NAM, 100 μM) did not show any changes in mitochondrial DNA (Fig. 4a). Additionally, RPTC exposed to SRT1720 for 24 hrs demonstrated elevations in ATP levels compared to vehicle cells (Fig. 4b). Pretreatment of RPTC with the synthetic SIRT1 inhibitor sirtinol (100 μM) or NAM prevented the SRT1720-mediated increased ATP levels at 24 hrs. The data from these experiments verify that SIRT1 is required for SRT1720-induced mitochondrial biogenesis.

AMPK, a primary energy regulator, monitors AMP/ATP levels and activates energy-producing mechanisms when this ratio is elevated (Hardie et al., 2003). AMPK regulates energy supply by directly phosphorylating modulators of metabolic pathways, including PGC-1α (Jager et al., 2007). Indeed, PGC-1α has at least two sites available for AMPK-mediated phosphorylation, and activators of SIRT1, such as resveratrol, can also induce activation of AMPK (Zang et al., 2006). To determine if SRT1720 also induces AMPK activation, RPTC were treated for 1 hr and 24 hr with SRT1720 or vehicle and activation of AMPK was detected by immunoblotting for phosphorylated AMPK (Thr172). AICAR and metformin were used concurrently as positive controls for AMPK activation. At both 1 hr and 24 hr, there was no effect on pAMPK levels by SRT1720, whereas a significant induction was observed with metformin treatment at both time points (Fig. 5). Contrary to previous reports in other systems (Zang et al., 2006), we did not observe any changes in pAMPK with AICAR treatment. Total AMPK levels did not change with any treatment. These data provide evidence that SRT1720 acts through SIRT1 activation and not concurrent activation of AMPK.

Because PGC-1α and mitochondrial biogenesis have a pivotal role in the recovery of RPTC from oxidant-induced mitochondrial dysfunction (Rasbach and Schnellmann, 2007a; Rasbach and Schnellmann, 2007b), we tested the hypothesis that pharmacological activation of mitochondrial biogenesis following injury would expedite recovery of mitochondrial functions in RPTC. RPTC were incubated with 400 μM tert-butyl hydroperoxide (TBHP) to induce oxidant injury. At 6 hr post-injury, RPTC were treated with SRT1720 to stimulate mitochondrial biogenesis. At 24 hrs, mitochondrial function

and cell morphology of injured RPTC treated with SRT1720 or vehicle were examined. Uncoupled respiration and ATP levels were approximately 60% of control in TBHP-injured RPTC at 24 hr (Fig. 6). In contrast, injured cells treated with SRT1720 demonstrated partial recovery of uncoupled respiration and full recovery of ATP levels 24 hrs post-injury (Fig. 6). Correlating with partial recovery of mitochondrial functions, recovery of RPTC morphology was observed in injured cells treated with SRT1720. Six hours after TBHP exposure, the injury was characterized by a loss of approximately 50% of cells as visualized by denuded areas of the dish as cells had sloughed off the plate surface, as well as a generalized shrinkage and rounding of adherent cells. RPTC treated with SRT1720 for 24 hrs following injury reverted to a pre-injury state characterized by reorganization and migration of surviving cells returning to a confluent monolayer and dome formation indicative of polarized RPTC (Fig. 7). This recovery was not as apparent in vehicle-treated injured cells. The data from these experiments indicate that SIRT1 activation can reverse oxidant-induced mitochondrial dysfunction, and recovery of mitochondrial numbers and function may aid in recovery of RPTC morphology following acute injury.



## DISCUSSION

Mitochondrial dysfunction is a common mechanism in the etiology of organ injuries and diseases characterized by metabolic insufficiency. Mitochondrial health is essential for cell and organ function due to their role in ATP production, fatty acid and lipid

metabolism, signaling pathways, and apoptosis. Despite potential for treating disorders characterized by mitochondrial impairment, very few therapies target the mitochondria to promote its function. In this study we demonstrated that pharmacologically-induced mitochondrial biogenesis enhanced mitochondrial function in RPTC and restored function following an acute injury.

SRT1720 stimulated mitochondrial biogenesis in RPTC within 24 hrs of exposure. Elevated levels of mitochondrial DNA, proteins, and function were observed with 10 μM treatment. The findings agree with results we have previously published linking isoflavone-induced mitochondrial biogenesis with SIRT1 activation (Rasbach and Schnellmann, 2008), as well as others who have demonstrated mitochondrial biogenesis with resveratrol in other cell types (Lagouge et al., 2006; Csiszar et al., 2009). The pharmacological advantage of SRT1720 over isoflavones is that SRT1720 produces mitochondrial biogenesis within 24 hrs, a key requirement if targeting acute organ injury.

While SRT1720 was previously reported as a SIRT1 activator, its mechanism of mitochondrial biogenesis in a cellular system is incomplete. Previous studies examining SRT1720-induced mitochondrial biogenesis have based their interpretations primarily on indirect mitochondrial measurements, such as respiration and ATP levels and ETC activity, predominantly in skeletal muscle cell lines (Feige et al., 2008; Smith et al., 2009). In this study, we sought to explore SRT1720-induced mitochondrial biogenesis in primary kidney cell cultures, which better mimic the metabolic properties of renal cells *in vivo*, not only by examining alterations in functional output, but also by examining direct

measurements of mitochondrial protein and DNA expression. When primary RPTC cultures were incubated with SRT1720, mitochondrial proteins NDUFB8 and ATP synthase β, and mitochondrial DNA copy numbers were elevated compared to vehicle-treated cells (Fig. 3), indicating mitochondrial biogenesis occurred within 24 hrs. Furthermore, we confirmed that elevations in mitochondrial components corresponded with increased mitochondrial output by examining cellular respiration and ATP production. Finally, we verified that the observed effects of SRT1720 were dependent on SIRT1 activity by using pharmacologic inhibitors of SIRT1 (Fig. 4), similar to what has been shown previously in other cell types using SIRT1 shRNA (Feige et al., 2008).

SIRT1 activation results in deacetylation of target proteins, and several substrates have been identified, including PGC-1α. SRT1720 elevated expression of deacetylated nuclear PGC-1α at 24 hrs in RPTC (Fig. 2a and 2b). The elevated expression was neither the result of increased PGC-1 transcription, as we did not observe any changes in PGC-1α mRNA expression (Fig. 2c), nor an increased resistance to proteasomal targeted degradation (Fig 2d). When SRT1720-treated cells were exposed to the inhibitor of protein translation cycloheximide, nuclear PGC-1α degraded at the same rate as vehicle cells, indicating the protein is still susceptible to proteasomal degradation (Sano et al., 2007; Rasbach et al., 2008). Taken together, these data indicate that SRT1720 did not induce PGC-1α transcription or increase stability of the protein. However, it is possible that the increased expression of nuclear PGC-1α may have been the result of an earlier transcriptional event that was missed by examining the 24 hr time point or may be the result of increased nuclear sequestration.

We did not observe any activation of AMPK with SRT1720 as examined by immunoblotting for Thr172-phosphorylated AMPK or in total AMPK (Fig. 5), which is consistent with previous reports that this compound exerts its effects in an AMPK-independent mechanism (Feige et al., 2008). Interestingly, metformin induced a robust phosphorylation of AMPK within 1 hr of treatment which was maintained for at least 24 hrs. In contrast AICAR, which has previously been shown to induce phosphorylation of AMPK in other cell types (Zang et al., 2006), did not have any effect on pAMPK expression after 1 or 24 hr treatment in RPTC. We have not explored the reason for the differential effects of AICAR in RPTC and other cell types

PGC-1α is an emerging therapeutic target for mitochondrial abnormalities due to its regulatory role in controlling metabolic processes and mitochondrial activities and biogenesis within the cell. Enhancing PGC-1α expression or activity has proven effective in reversing the phenotypic consequences of mitochondrial impairment. Mitochondrial myopathies can be rescued through transgenic expression of PGC-1α or the pharmacologic PPAR pan-agonist bezafibrate, both of which induce mitochondrial biogenesis, enhance respiratory capacity, conserve ATP levels, and prolong lifespan (Wenz et al., 2008). Pharmacological stimulation or adenoviral upregulation of PGC-1α rescued mitochondrial function and bioenergetics and restored insulin signaling in insulin-resistant skeletal muscle cells (Pagel-Langenickel et al., 2008). Finally, the benefits of exercise and caloric restriction to rescue or protect against metabolic

deficiencies has been linked to enhanced PGC-1α activity (Koves et al., 2005; Boily et al., 2008).

Recent evidence indicates that induction of PGC-1α and mitochondrial biogenesis is a critical adaptive response to maintain energy levels and metabolic demands required during recovery from certain acute injuries to cells and organs (Rasbach and Schnellmann, 2007b; Wang et al., 2008; Yin et al., 2008). In response to partial hepactectomy, C/EBPβ transcriptionally induces PGC-1α in order to maintain metabolic homeostasis and energy demands of the regenerating liver (Wang et al., 2008). In response to oxidant-induced mitochondrial dysfunction in RPTC, induction of PGC-1α and mitochondrial biogenesis is an adaptive repair mechanism initiated by the cell, which can be stimulated by PGC-1α over-expression (Rasbach and Schnellmann, 2007a; Rasbach and Schnellmann, 2007b). Here, we show that pharmacologically-induced mitochondrial biogenesis also rescues mitochondrial functions following oxidant-induced injury. Within 24 hrs SRT1720 reversed mitochondrial dysfunction and ATP depletion resulting from TBHP toxicity (Fig. 6).

Although the majority of studies investigating PGC-1α-mediated mitochondrial regulation through AMPK or SIRT1 are focusing on its role in chronic or age-related metabolic deficiencies (Guarente, 2007; Milne and Denu, 2008), this pathway offers a unique target for the treatment of acute organ injuries that are also plagued by mitochondrial impairment. As observed in this study as well as previous reports (Rasbach and Schnellmann, 2007a; Rasbach and Schnellmann, 2007b), mitochondrial

biogenesis has a pivotal role in recovery of critical mitochondrial functions in oxidant-injured renal cells. Acute organ injuries, such as ischemic acute kidney injury (AKI), are characterized by de-energization of the mitochondria as well as loss of mitochondrial proteins and depletion of cellular energy stores (Weinberg et al., 2000; Bonventre and Weinberg, 2003; Feldkamp et al., 2005), which could exacerbate cell death and organ failure or limit energy-dependent repair processes if mitochondrial function is not restored. These studies provide evidentiary basis to study the involvement of mitochondrial repair processes in the recovery from organ injuries such as AKI, and highlights the therapeutic potential of pharmacological inducers of mitochondrial biogenesis to rescue mitochondrial function in injuries and disorders plagued by mitochondrial impairment.

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## FOOTNOTES

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## LEGENDS FOR FIGURES

**Figure 1. SRT1720 enhances SIRT1 deacetylase activity.** Recombinant SIRT1 enzyme was incubated with acetylated peptide, $NAD^+$ and various concentrations of SRT1720. Increasing concentrations of SRT1720 corresponded with increased SIRT1 activity, which was measured indirectly from a fluorescent signal produced that was relative to levels of deacetylated product. Data are presented as mean % control +/- SEM. Different superscripts indicate data are significantly different from each other ($p<0.05$).

**Figure 2. Nuclear deacetylated PGC-1α expression is elevated in SRT1720-treated cells.** *a*, Nuclear lysates were fractionated from vehicle and SRT1720-treated cells and PGC-1 expression was assessed by immunoblot analysis. Histone H3 expression verified equal protein input within sample groups. *b*, The acetylation state of PGC-1α was examined in nuclear lysates by immunoprecipitating PGC-1α followed by immunoblot analysis of acetylated lysine residues and PGC-1α. Because SRT1720 lysates contained more PGC-1α, supernatants from immunoprecipitations were subjected to Histone H3 immunoblot analysis for input control. *c*, PGC-1α mRNA expression in SRT1720 and vehicle cells was determined by real-time PCR using primers designed to measure PGC-1α transcripts and GAPDH as internal control. *d*, PGC-1α degradation was examined in SRT1720 cells by extracting nuclear protein at 0, 30, and 60 min following cycloheximide exposure and immunoblotting for PGC-1α expression. Histone H3

expression verified protein input. Data are presented as mean % control +/- SEM. Different superscripts indicate data are significantly different from each other ($p<0.05$).

**Figure 3. SRT1720 induces mitochondrial biogenesis in RPTC within 24 hrs.** *a,* Mitochondrial DNA copy numbers were assessed by real-time PCR. DNA isolated from RPTC treated with vehicle or 10 μM SRT1720 was analyzed by real-time PCR for relative quantities of the mitochondrial gene ND6 and the nuclear gene Pou5f1. *b*, Mitochondrial proteins ATP Synthase β and NDUFB8 were measured by immunoblot analysis in cells treated with 1, 3, or 10 uM SRT1720. GAPDH immunoblots were performed to verify equal protein input. *c*, Mitochondrial function was assessed in vehicle and SRT1720-treated cells. Basal and FCCP-uncoupled respiration and ATP levels were measured in RPTC treated with 1, 3, or 10 uM SRT1720. Total protein was measured by BCA and used for normalization of data. Data are presented as mean % control +/- SEM. Different superscripts indicate data are significantly different from each other ($p<0.05$).

**Figure 4. SRT1720-induced mitochondrial biogenesis is SIRT1-dependent.** *a,* RPTC were treated with 10 μM SRT1720 alone or in the presence of the SIRT1 inhibitor nicotinamide (100 μM NAM) for 1 hr prior to SRT1720 addition. Mitochondrial DNA levels were analyzed by real-time PCR for the mitochondrial gene ND6. The nuclear encoded gene Pou5f1 was used for normalization. *b*, RPTC were treated with SRT1720 alone or in combination with the SIRT1 inhibitors nicotinamide or sirtinol. ATP levels were measured 24 hrs after SRT1720 addition. Total protein was measured by BCA and

used for normalization. Data are presented as mean % control +/- SEM. Different superscripts indicate data are significantly different from each other ($p<0.05$).

**Figure 5. SRT1720 does not activate AMP-dependent kinase (AMPK).** RPTC treated with 10 μM SRT1720 or vehicle for 1 or 24 hrs were subjected to immunoblot analysis using antibodies to detect phosphorylated AMPK (Thr172), total AMPK, and GAPDH. The known AMPK activators AICAR (500 μM) and metformin (1 mM) were used as positive controls for pAMPK antibody. GAPDH expression was analyzed for load control. Different superscripts indicate data are significantly different from each other ($p<0.05$).

**Figure 6. Mitochondrial function is rescued in SRT1720-treated cells following oxidant injury.** RPTC injured with the oxidant *tert*butyl hydroperoxide (TBHP) were treated with 10 μM SRT1720 or an equal volume of DMSO 6 hrs after injury and ATP levels and FCCP-uncoupled respiration were measured 24 hrs post-injury. Total protein was measured by BCA for data normalization. Data are presented as mean % control +/- SEM. Different superscripts indicate data are significantly different from each other ($p<0.05$).

**Figure 7. RPTC morphology is partially recovered in cells treated with SRT1720 following TBHP toxicity.** RPTC exposed to 400 μM TBHP were treated with 10 μM SRT1720 or DMSO 6 hrs after injury and then examined by light microscopy (10 X magnification) for changes in cell morphology at 24 hrs post-injury.

Figure 1

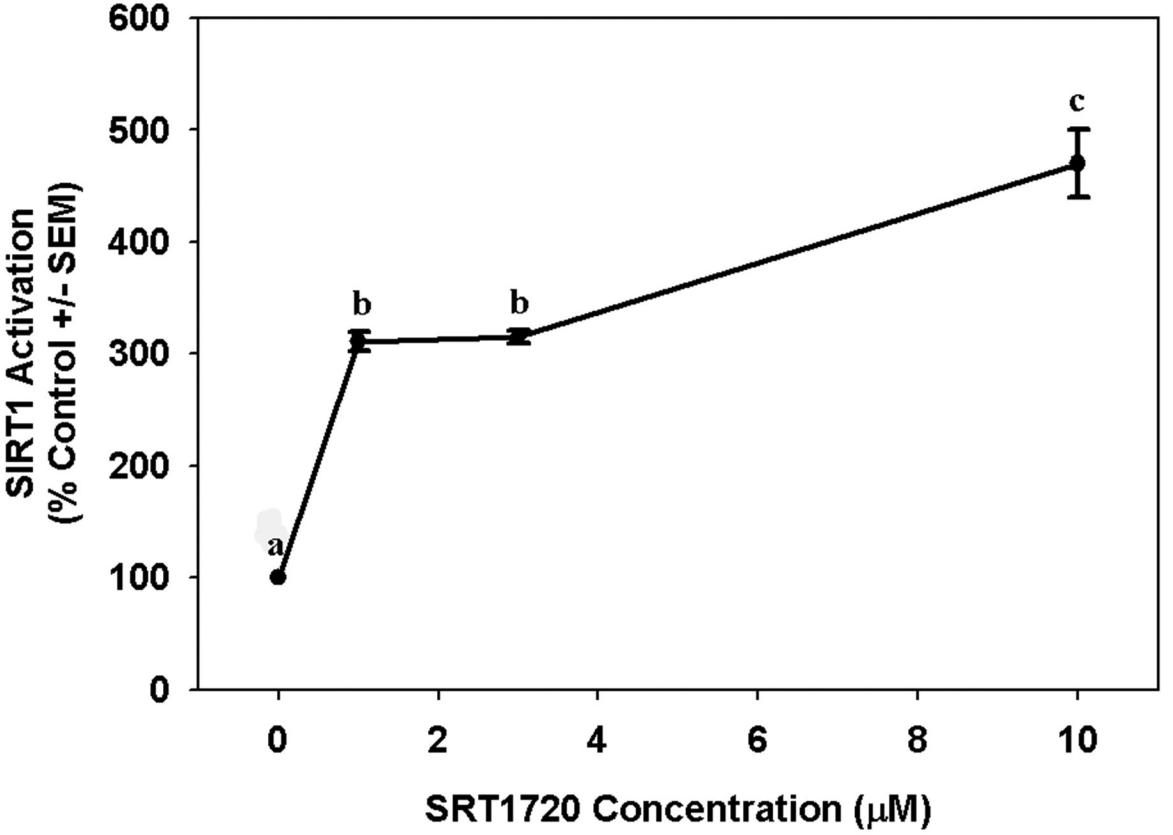

600
500
400
300
200
100
0
SIRT1 Activation
(% Control +/- SEM)
a
b
b
c
0
2
4
6
8
10
SRT1720 Concentration (μM)

Figure 2

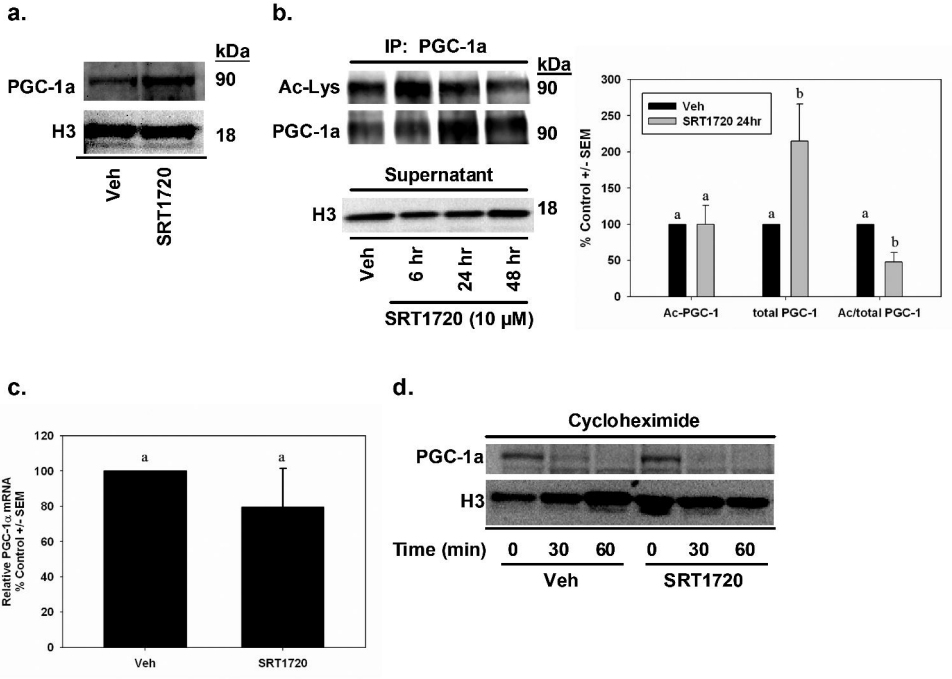
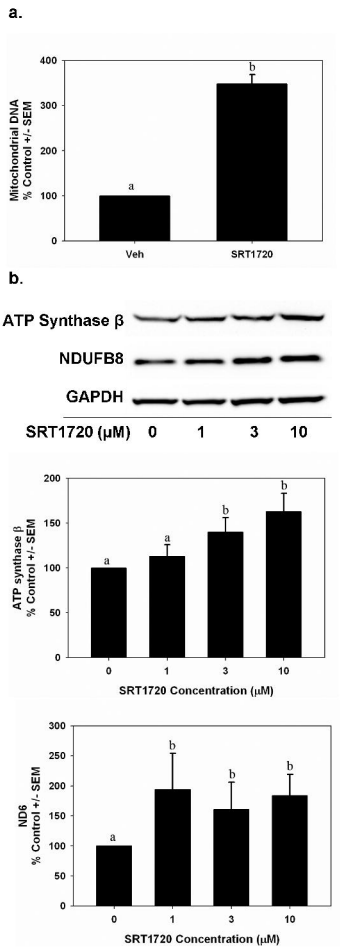
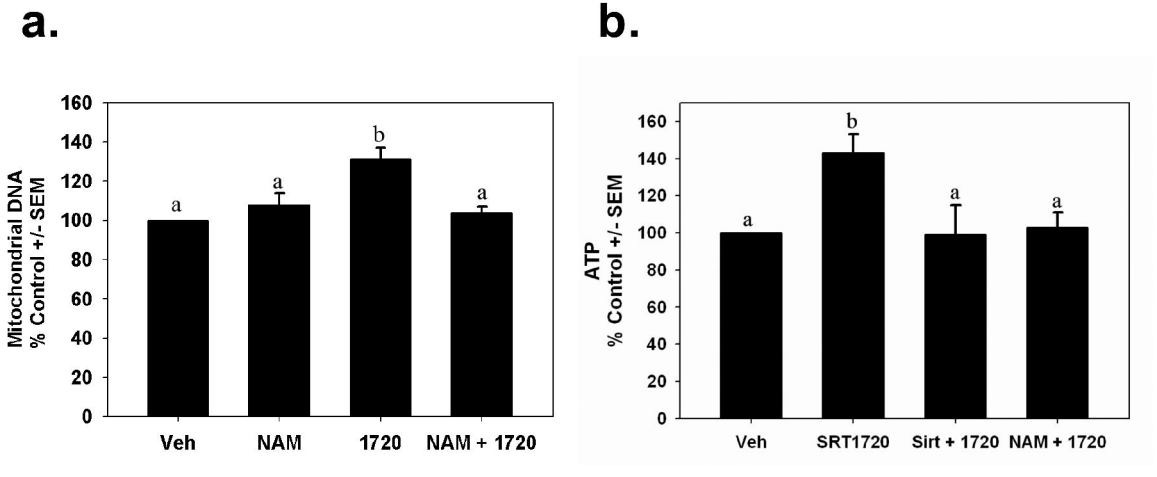

a.
kDa
PGC-1a
90
H3
18
Veh
SRT1720
b.
IP: PGC-1a
kDa
Ac-Lys
90
PGC-1a
90
Supernatant
H3
18
Veh
6 hr
24 hr
48 hr
SRT1720 (10 μM)
% Control +/- SEM
Veh
SRT1720 24hr
Ac-PGC-1
total PGC-1
Ac/total PGC-1
c.
Relative PGC-1α mRNA
% Control +/- SEM
Veh
SRT1720
d.
Cycloheximide
PGC-1a
H3
Time (min)
0
30
60
0
30
60
Veh
SRT1720

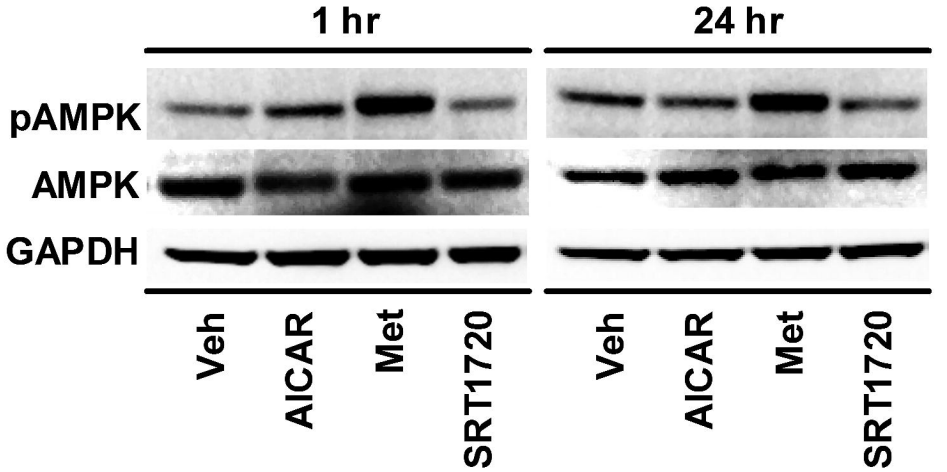
Figure 3

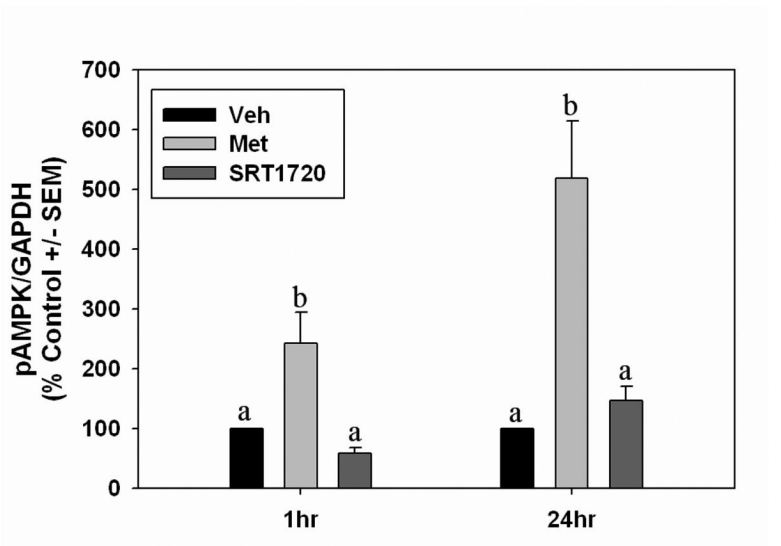

a.
Mitochondrial DNA
% Control +/- SEM
400
300
200
100
0
a
b
Veh
SRT1720
b.
ATP Synthase β
NDUFB8
GAPDH
SRT1720 (μM) 0 1 3 10
ATP synthase β
% Control +/- SEM
200
150
100
50
0
a
a
b
b
0
1
3
10
SRT1720 Concentration (μM)
ND6
% Control +/- SEM
300
250
200
150
100
50
0
a
b
b
b
0
1
3
10
SRT1720 Concentration (μM)
c.
Basal Respiration
% Control +/- SEM
180
160
140
120
100
80
60
40
20
0
a
b
b
b
0
1
3
10
SRT1720 Concentration (μM)
Uncoupled Respiration
% Control +/- SEM
200
150
100
50
0
a
a
b
b
0
1
3
10
SRT1720 Concentration (μM)
ATP
% Control +/- SEM
250
200
150
100
50
0
a
b
b
b
0
1
3
10
SRT1720 Concentration (μM)

# Figure 4

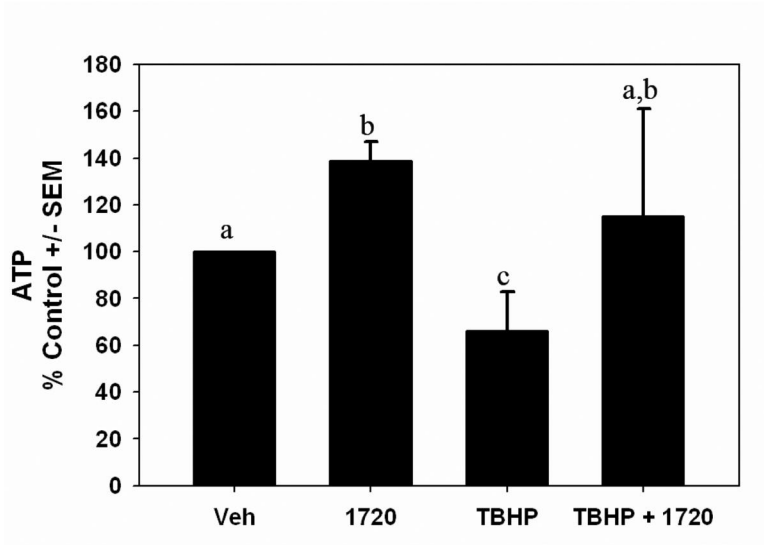

a.
b.
Mitochondrial DNA
% Control +/- SEM
ATP
% Control +/- SEM
0
20
40
60
80
100
120
140
160
a
a
b
a
Veh
NAM
1720
NAM + 1720
a
b
a
a
Veh
SRT1720
Sirt + 1720
NAM + 1720

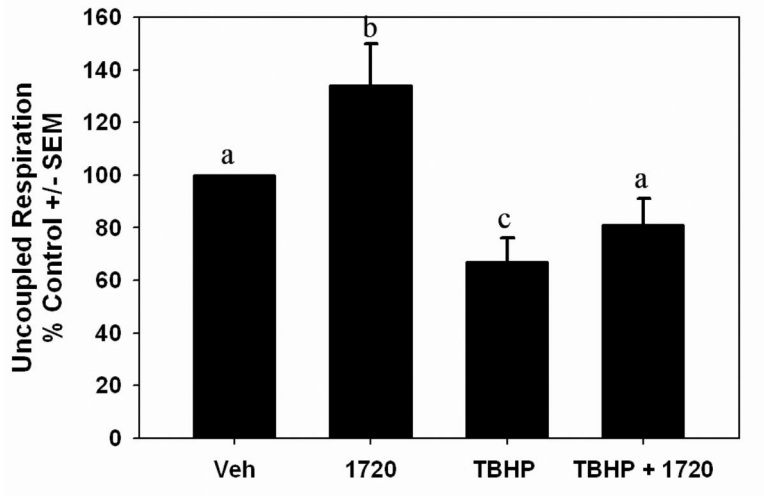
# Figure 5


1 hr
24 hr
pAMPK
AMPK
GAPDH
Veh
AICAR
Met
SRT1720
Veh
AICAR
Met
SRT1720


pAMPK/GAPDH
(% Control +/- SEM)
700
600
500
400
300
200
100
0
Veh
Met
SRT1720
a
b
a
a
b
a
1hr
24hr

# Figure 6


ATP
% Control +/- SEM
180
160
140
120
100
80
60
40
20
0
a
b
c
a,b
Veh
1720
TBHP
TBHP + 1720


Uncoupled Respiration
% Control +/- SEM
160
140
120
100
80
60
40
20
0
a
b
c
a
Veh
1720
TBHP
TBHP + 1720

# Figure 7