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Title page

cGMP-Selective Phosphodiesterase Inhibitors Stimulate Mitochondrial Biogenesis and Promote Recovery from AKI

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Running title page

Running title: PDE Inhibitors Stimulate MB and Recovery from AKI

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Nonstandard abbreviations: AKI, acute kidney injury; ATPSβ, ATP synthase subunit beta; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; CREB, cAMP-response element binding protein; COX1, cytochrome c oxidase subunit 1; DOI, 1-(2, 5-dimethoxy-4-iodophenyl)-2-aminopropane hydrochloride; eNOS, endothelial nitric oxide synthase; FA, folic acid; FCCP, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone; MB, mitochondrial biogenesis; mtDNA, mitochondrial DNA; ND1, NADH dehydrogenase 1; ND6, NADH dehydrogenase 6, NDUFβ8, NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 8; Nrf1, nuclear respiratory factor 1; Nrf2, nuclear respiratory factor 2; NO, nitric oxide; OCR, oxygen consumption rate; PDE3, phosphodiesterase 3; PDE4, phosphodiesterase 4; PDE5, phosphodiesterase 5; PGC-1α, peroxisome proliferator-activated receptor γ coactivator-1 alpha; PKA, protein kinase A; RNS, reactive nitrogen species; ROS, reactive oxygen species; RPTC, renal proximal tubular cells; SIRT1, silent mating type information regulation 2 homolog 1; Tfam, mitochondrial transcription factor A

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Abstract

Recent studies demonstrated that mitochondrial dysfunction is a mediator of acute kidney injury (AKI). Consequently, restoration of mitochondrial function following AKI may be key to the recovery of renal function. Mitochondrial function can be restored through the generation of new, functional mitochondria in a process called mitochondrial biogenesis (MB). Despite its potential therapeutic significance, very few pharmacological agents have been identified to induce MB. To examine the efficacy of phosphodiesterase 3 (PDE3, cAMP and cGMP activity) and 4 (PDE4, cAMP activity) inhibitors in stimulating MB, primary cultures of renal proximal cells (RPTC) were treated with a panel of inhibitors for 24h and PDE3, but not PDE4 inhibitors, increased FCCP-uncoupled oxygen consumption rate (FCCP-OCR), a marker of MB. Exposure of RPTC to the PDE3 inhibitors, cilostamide and trequinsin, for 24h increased peroxisome proliferator-activated receptor γ coactivator-1 alpha (PGC-1α), and multiple mitochondrial electron transport chain genes. Cilostamide and trequinsin also increased mRNA expression of mitochondrial genes and mtDNA copy number in mice renal cortex. Consistent with these experiments, 8-Br-cGMP increased FCCP-uncoupled OCR and mitochondrial gene expression, while 8-Br-cAMP had no effect. The cGMP-specific PDE5 inhibitor sildenafil also induced MB in RPTC and in vivo in mouse renal cortex. Treatment of mice with sildenafil after folic acid (FA)-induced AKI promoted restoration of MB and renal recovery. These data provide strong evidence that specific PDE inhibitors that increase cGMP are inducers of MB in vitro and in vivo, and suggest their potential efficacy in AKI and other diseases characterized by mitochondrial dysfunction and suppressed MB.

Introduction

Mitochondrial dysfunction is increasingly recognized as an important pathophysiological mediator of a variety of disease states including neurodegeneration, cardiovascular disease, metabolic syndrome, and acute organ injury (Hwang; Choumar et al., 2011; Yan et al., 2011; Pundik et al., 2012; Andreux et al., 2013; Bayeva et al., 2013; Cheng and Ristow, 2013; Cooper, 2013). Mitochondrial dysfunction is an established component of the pathogenesis of acute kidney injury (AKI) and cause of renal tubular dysfunction and cell death (Jassem et al., 2002; Jassem and Heaton, 2004; Hall and Unwin, 2007; Weinberg, 2011; Venkatachalam and Weinberg, 2012). Our group has demonstrated persistent disruption of mitochondrial homeostasis and inhibition of mitochondrial biogenesis (MB) following ischemia-reperfusion (I/R), rhabdomyolysis-induced (Funk and Schnellmann, 2012) and folic acid (FA)-induced (unpublished data) AKI . Restoration of mitochondrial number and function is thought to be required for recovery from AKI due to the high energy requirements of tissue repair. These data provide support for development of pharmacological agents that induce MB for treatment of AKI and other pathologies characterized by mitochondrial dysfunction.

Mitochondria are dynamic organelles that are continuously regenerated through the processes of biogenesis, mitophagy, fission and fusion (Brooks et al., 2009; Shaw and Winge, 2009; Cho et al., 2010; Funk and Schnellmann, 2012; Kubli and Gustafsson, 2012). MB is the assembly of new mitochondria from existing mitochondria, occurring under basal conditions to replace damaged mitochondria, but is rapidly induced in response to both physiological and pathophysiological stimuli including sepsis, exercise, fasting, hypoxia and cellular injury (Puigserver and Spiegelman, 2003; Tran et al., 2011; Kang and Li Ji, 2012; Wenz, 2013). The primary regulator of MB is the transcriptional co-activator peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α). PGC-1α exerts its functions by activating the transcription factors, nuclear respiratory factors 1 and 2 (Nrf1 and Nrf2). Nrf1 controls the expression of

mitochondrial transcription factor A (Tfam), which regulates the transcription of mtDNA (Puigserver et al., 1998; Wu et al., 1999; Scarpulla, 2008; Scarpulla et al., 2012). PGC-1α is enriched in tissues with high metabolic demand, including heart, muscle and kidneys (Liang and Ward, 2006). The ability of PGC-1α to respond to a variety of stimuli and its importance in cellular bioenergetics make it an ideal target for pharmacological intervention in disease states characterized by mitochondrial disruption.

Despite the promise of PGC-1α and MB as a therapeutic target, there is a paucity of pharmacological agents capable of stimulating PGC-1α expression and activity. Activators of silent mating type information regulation 2 homolog 1 (SIRT1) including isoflavones, resveratrol and SRT1720 have been demonstrated to induce PGC-1α and promote increased mitochondrial number and function (Rasbach and Schnellmann, 2008; Funk et al., 2010; Menzies et al., 2013). Our laboratory also identified the 5-hydroxytryptamine type 2 agonist, DOI, and the β_2-adrenergic receptor agonist, formoterol, as potent inducers of PGC-1α and MB (Rasbach et al., 2010; Wills et al., 2012). Stimulation of MB following injury accelerates recovery of cellular morphology and function (Rasbach and Schnellmann, 2007; Funk et al., 2010; Rasbach et al., 2010). These data demonstrate the importance of MB in recovery of renal tubular epithelial cells following injury and suggest agents that stimulate MB could serve as viable therapies following AKI.

Due to the importance of the cAMP/PKA/CREB axis in PGC-1α regulation, drugs that increase cellular cAMP levels may induce MB. The β_2-adrenergic signaling cascade, which upon activation increases intracellular cAMP through G_s mediated activation of adenylyl cyclase, has been shown to regulate oxidative metabolism and energy expenditure (Tadaishi et al., 2011; Muller et al., 2013). Formoterol induces MB in RPTC, and mice treated with formoterol demonstrated increased mitochondrial gene expression and mtDNA copy number in renal cortex and heart (Wills et al., 2012). cGMP levels have also been shown to regulate PGC-1α

expression and MB. Pharmacologically-induced generation of NO via eNOS and subsequent NO-dependent activation of guanylyl cyclase led to MB in U937, L6 and PC12 cells. (Nisoli et al., 2004).

Both cAMP and cGMP levels are tightly regulated through cleavage to AMP and GMP, respectively, by a class of enzymes called cyclic nucleotide phosphodiesterases (PDEs). The PDE superfamily consists of 11 families differing in tissue distribution, regulation and substrate affinity (e.g. cAMP vs. cGMP) (Francis et al., 2011). Potent, selective inhibitors of nearly all family members are available. (Bender and Beavo, 2006). Inhibition of phosphodiesterases would serve as a novel, and potentially efficacious drug target to induce MB. As such, we studied inhibitors of PDE3, PDE4 and PDE5 for their ability to induce MB in the kidney, and promote recovery from FA-induced AKI.

Materials and Methods

Reagents. Cilostamide, trequinsin, (R)-(-)-rolipram, Ro 20-1724, sildenafil, 8-Br-cAMP and 8-Br-cGMP were purchased from Tocris Bioscience (Ellisville, MO). All other chemicals were obtained from Sigma Aldrich (St. Louis, MO).

Animal Care and Use. Studies were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All protocols were approved by the Institutional Animal Care and Use Committee at the Medical University of South Carolina and all efforts were made to minimize animal suffering.

Isolation and Culture of Proximal Tubules. Female New Zealand white rabbits (1.5-2.0 kg) were purchased from Charles River Laboratories (Wilmington, MA). Renal proximal tubules cells (RPTC) were isolated using the iron oxide perfusion method described previously (Nowak and Schnellmann, 1995). For respirometry experiments, cells were plated on 100-mm culture grade Petri dishes and at 37^{o}C in a 5% CO_2/95% air environment. Dishes were continuously swirled on an orbital shaker at 80 RPM. Cell culture media consisted of a 1:1 mixture of Dulbecco's modified Eagle's essential medium and Ham's F-12 (lacking glucose, phenol red and sodium pyruvate; Invitrogen, Carlsbad, CA), supplemented with HEPES (15 mM), glutamine (2.5 mM), pyridoxine HCl (1 μM), 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 media. After 3 days of culture, de-differentiated RPTC were trypsinized and replated on XF-96 polystyrene cell culture microplates (Seahorse Bioscience, North Bellerica, MA) at a concentration of 18,000 cells/well. Cells were maintained at 37^{o}C for an additional 2 days before experimentation (Beeson et al., 2010). For all other RPTC experiments, cells were plated and cultured in 35-mm dishes in the media described above. Experiments were performed on the sixth day after

plating when cells had formed a confluent monolayer. RPTC were treated with various compounds for 24h unless otherwise noted.

Oxygen Consumption. The oxygen consumption rate (OCR) of RPTC was measured using the Seahorse Bioscience XF-96 Extracellular Flux Analyzer according to the protocol described previously (Beeson et al., 2010). Each assay plate was treated with vehicle control (DMSO <0.5%), and increasing concentrations of the compounds of interest. Basal OCR was measured before injection of carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP, 0.5 μM), allowing for the measurement of uncoupled OCR.

Testing of compounds in C57BL/6 Mice. Male C57BL/6 mice (6-8 weeks old) were obtained from the National Cancer Institute (Bethesda, MD). Mice were housed individually in a temperature-controlled room under a 12-h light/dark cycle. Mice were randomly assigned to saline control, cilostamide (0.3 mg/kg or 3 mg/kg), trequinsin (0.3 mg/kg or 3 mg/kg) or sildenafil (0.3 mg/kg or 3 mg/kg) treatment groups. Mice were given a single intraperitioneal injection of saline or compound at the doses described above. Mice were euthanized by CO_2 asphyxiation followed by cervical dislocation 24h after treatment. Kidneys were removed and preserved by flash-freezing in liquid nitrogen. Tissues were stored at -80^{o}C for later analysis.\

Folic Acid Animal Model. Male C57BL/6 mice (8-10 weeks of age) were given a single intraperitoneal injection of 250 mg/kg folic acid dissolved in 250mM sodium bicarbonate or saline control based on previous literature (Doi et al., 2006). Mice were injected with sildenafil (0.3 mg/kg) or diluent every 24 hours beginning 1d after FA injection. Mice were euthanized at 7d via isoflurane asphyxiation and cervical dislocation. Kidneys were removed and preserved by flash-freezing.

Quantitative Real-Time PCR. Total RNA was extracted from RPTC or renal cortex samples using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. cDNA was

synthesized via reverse transcription using the iScript Advanced cDNA synthesis kit (Biorad, Hercules, CA) with 2 µg of RNA. qPCR was performed with cDNA using SsoAdvanced SYBR Green Supermix (Bio-Rad) at a concentration of 1X and primers at a concentration of 750 nM (Integrated DNA Technologies, Coralville, IA). mRNA expression of all genes was calculated using the 2-ΔΔCT method normalized to tubulin in RPTC or β-actin in renal cortical tissue. Primer sequences for PGC-1α, ND6, NDUFβ8, and tubulin were described previously (Funk and Schnellmann, 2012). Primer sequences for ND1 and β-actin were as follows: ND1: sense 5'-TAGAACGCAAAATCTTAGGG-3', antisense 5'-TGCTAGTGTGAGTGATAGGG-3'; β-actin: sense 5'- GGGATGTTTGCTCCAACCAA-3', antisense 5'-GCGCTTTTGACTCAAGGATTTAA-3'.

Mitochondrial DNA Content. Real-time PCR was used to determine the relative quantity of mitochondrial DNA (mtDNA) in both RPTC and mouse renal cortical tissue samples. After treatment, DNA was extracted from cells or tissue using the DNeasy Blood and Tissue Kit (QIAGEN, Valencia, CA) and 5 ng of cellular DNA was used for qPCR. For RPTC samples, mitochondrial encoded NADH dehydrogenase 6 (ND6) was used to measure mitochondrial copy number and was normalized to nuclear-encoded tubulin expression. For renal cortex, NADH dehydrogenase 1 (ND1) was used as the mitochondrial gene and expression was normalized to nuclear-encoded β-actin expression.

cAMP and cGMP ELISA. RPTC in 35-mm dishes were treated with vehicle control (DMSO) or compound of interest for 20 min. RPTC were then harvested according to manufacturer's protocol and cAMP or cGMP levels were measured using a commercially available ELISA kit (Cayman Chemical, Ann Arbor, MI).

Tissue ATP levels. ATP was isolated from renal cortical tissue via phenol-TE extraction as previously described (Chida et al., 2012). Briefly, freshly prepared tissue was homogenized in

3.0 mL of ice-cold TE saturated phenol. One mL of the homogenate was combined with 200 µL chloroform and 150 µL of deionized water, vortexed and centrifuged at 10,000xg for 5 min at 4^{o}C. An aliquot from the supernatant was diluted 200-fold in deionized water, and ATP levels were measured using a luciferin-luciferase based ATP determination kit (Invitogen).

Statistical Analysis. Data are presented as means ± SEM. Single comparisons were performed using a Student's t-test. Multiple comparisons were subjected to one-way analysis of variance (ANOVA) followed by a Student-Newman-Keuls test with $p<0.05$ considered to be a statistically significant difference between means. RPTC isolated from a single rabbit represented an individual experiment (n=1) and were repeated until n≥4 was obtained. Mouse studies were repeated until n≥3 was obtained.

Results

PDE3 inhibitors, but not PDE4 inhibitors increase FCCP-uncoupled OCR in RPTC.

We treated RPTC in XF-96 culture plates with the PDE3 inhibitors cilostamide or trequinsin, the PDE4 inhibitors (R)-(-)-rolipram or Ro 20-1724, or vehicle control for 24h. PDE3 hydrolyzes both cAMP and cGMP to their non-cyclic forms, AMP and GMP, while PDE4 specifically hydrolyzes cAMP to AMP (Francis et al., 2011). FCCP-OCR increased in RPTC compared to vehicle control after a 24h exposure to cilostamide (25-100 nM) and trequinsin (30-100 nM) (Fig. 1A and 1B), but no significant changes were observed in FCCP-OCR following treatment with (R)-(-)-rolipram (0.5-50 μM) or Ro 20-1724 (5-20 μM) (Fig. 1C and 1D). These data suggest a functional selectivity for the MB response between PDE3 and PDE4 inhibition in RPTC.

PDE3 inhibitors induce MB in RPTC. To validate that the increased FCCP-OCR observed in RPTC following treatment with PDE3 inhibitors was due to MB, mRNA levels for PGC-1α, the mitochondrial-encoded complex I protein ND6 and the nuclear-encoded complex I protein NDUFβ8 were measured via qPCR. Gene expression was normalized to tubulin. PGC-1α levels increased vs. control following treatment with cilostamide (1.8-fold) or trequinsin (2.5-fold) (Fig. 2). Additionally, mRNA expression of mitochondrial-encoded ND6 and the nuclear-encoded NDUFβ8 mitochondrial proteins were increased vs. control with cilostamide (1.5-fold and 2.2-fold, respectively) and trequinsin (1.8-fold and 2.4-fold, respectively). These data provide strong evidence that inhibition of PDE3 causes functional MB in RPTC.

Increased cGMP, but not cAMP induces MB in RPTC. To examine the functional selectivity of PDE3 and PDE4 inhibitors under conditions that induce MB, RPTC were treated with the PDE3 inhibitors cilostamide and trequinsin, the PDE4 inhibitor (R)-(-)-rolipram or vehicle control for a period of 20 minutes. Sildenafil, a specific inhibitor of PDE5 (cGMP-specific PDE) was included as a control. Both cAMP and cGMP levels increased in response to cilostamide and

trequinsin compared to controls treatments, while cAMP only increased in RPTC treated with rolipram (Fig. 3A and 3B). RPTC treated with sildenafil resulted in increased cGMP, but not cAMP. These data agree with the classical mechanisms of PDE3 (hydrolyzes both cAMP and cGMP), PDE4 (hydrolyzes only cAMP) and PDE5 (hydrolyzes only cGMP) (Bender and Beavo, 2006). The inability of rolipram and other PDE4 inhibitors tested to induce MB suggests that cGMP may be the primary mediator of MB in RPTC.

To test this hypothesis we treated RPTC with the cell permeable cyclic nucleotide analogs, 8-Br-cAMP and 8-Br-cGMP for a period of 24h. RPTC treated with 8-Br-cGMP (10-300 μM) showed a ~20% increase in FCCP-uncoupled OCR at all concentrations tested, while treatment with 8-Br-cAMP resulted in no change (Fig. 3C). To validate that this increase in FCCP-OCR is due to stimulation of MB, mRNA expression of PGC-1α, ND6 and NDUFB8 were measured by qPCR. RPTC treated with 8-Br-cGMP had elevated mRNA levels of PGC-1α (2.2-fold), ND6 (1.7-fold) and NDUFB8 (1.9-fold). 8-Br-cAMP had no effect on mitochondrial genes expression (Fig. 3D). Furthermore, to test the ability of a PDE5 inhibitor to induce MB *in vitro* RPTC were treated with sildenafil for 24h (1 nM – 1 μM) and FCCP-OCR was measured using the Seahorse XF96. RPTC treated with sildenafil showed a ~20% increase in FCCP-uncoupled OCR vs. control (Fig. 4A) at 10 and 100 nM. To validate that the increase in respiration was due to MB, mRNA levels of PGC-1α, ND6 and NDUFB8 were measured and found to increase 1.8-, 2.0- and 1.5-fold respectively (Fig. 4B).

PDE3 inhibitors induce MB in mouse renal cortex. In kidneys of mice treated with cilostamide, PGC-1α was induced 2- and 2.7-fold at doses of 0.3 and 3 mg/kg, respectively. Trequinsin induced PGC-1α 2.7- and 2.8- fold in the kidney at doses of 0.3 and 3 mg/kg. mRNA expression of the nuclear-encoded mitochondrial genes NDUFB8 and ATPSβ both increased greater than 2-fold in kidneys of mice treated with either cilostamide or trequinsin at 0.3 or 3 mg/kg (Fig. 5A, 5B). The mitochondrial-encoded mitochondrial genes NADH dehydrogenase 1

(ND1) and cytochrome C oxidase subunit I (COX1) increased in the kidneys of these mice. The mtDNA copy number was also increased in the kidneys of mice treated with cilostamide at 0.3 mg/kg, while mice treated with 3 mg/kg cilostamide had no effect. (Fig. 5C) Trequinsin increased mtDNA copy number in the kidneys 1.6- and 2-fold at doses of 0.3 and 3 mg/kg, respectively. (Fig. 5D) These data provide strong evidence that pharmacological inhibition of PDE3 induces MB in the kidney of naïve mice.

Sildenafil induces MB in mouse renal cortex. The selectivity of the MB response for cGMP in RPTC indicates that inhibitors of cGMP-specific PDE such as PDE5, may in fact be a better therapeutic target and could eliminate off-target effects due to the accumulation of cAMP. PDE5 inhibitors also have a much more favorable safety protocol than PDE3 inhibitors particularly for extended administration (Cruickshank, 1993).

To determine whether PDE5 inhibition is capable of inducing MB in the kidney *in vivo*, mice were given a single intraperitoneal injection of sildenafil (0.3 mg/kg or 3 mg/kg) or saline control. Mice were sacrificed and kidneys were harvested 24h after treatment. mRNA levels of PGC-1α, NDUFB8, ND1, ATPβ and COX1 were measured by quantitative real time PCR. All mitochondrial genes were increased versus saline treated animals except for COX1 and ATPSβ in mice treated with sildenafil at 3 mg/kg (Fig 6A). mtDNA copy number was assessed by qPCR in kidneys of sildenafil treated mice and was found to increase 1.6-fold in mice treated with 0.3 mg/kg sildenafil. No change in mtDNA copy number was observed in mice treated with 3 mg/kg sildenafil (Fig 6B).

To assess whether sildenafil-induced MB increased mitochondrial function in the kidney cortex, we measured ATP levels. ATP levels increased 32% in mice treated with 0.3 mg/kg sildenafil compared to control mice (Fig 6C). These data strongly support our hypothesis that PDE5 inhibitors induce MB and mitochondrial function *in vitro* and *in vivo.*

Sildenafil promotes recovery of MB and renal function after FA-induced AKI.

To test the hypothesis that sildenafil-induced MB will accelerate recovery of mitochondrial and renal function after AKI, we induced AKI by injecting FA and then treated these mice with sildenafil or vehicle once daily starting at 24h after injury for 6d. mRNA expression of COX1 and Tfam were reduced to 27% and 36% of control, respectively, in FA treated mice receiving vehicle control at 6d. Sildenafil-treated FA mice demonstrated a 1.6-fold increase in mRNA COX1 expression to 43% of control mice, and a 1.4-fold increase in Tfam expression to 50% of control (Fig 7A). mtDNA copy number was reduced to 36% of animals receiving FA alone, and treatment with sildenafil caused a ~2-fold induction to 63% of control. (Fig 7B). These data demonstrate that sildenafil can induce MB in a model of AKI.

To examine whether MB promoted renal recovery, KIM-1, a specific marker of tubular injury, was measured in renal cortex. KIM-1 levels were markedly increased (~6-fold) in FA treated animals compared to control animals and treatment of FA mice with sildenafil restored KIM-1 expression to control levels (Fig 7C, D). These data demonstrate that sildenafil promotes renal recovery with its induction of mitochondrial gene expression and mtDNA copy number.

Discussion

Mitochondria are highly regulated organelles, whose function is tightly linked to the metabolic demands and health of a cell (Brooks et al., 2009; Shaw and Winge, 2009; Funk and Schnellmann, 2012; Kubli and Gustafsson, 2012). Mitochondrial function is necessary for normal cell and tissue function, and critical in energy-dependent repair processes. A wide array of disease states are characterized by mitochondrial dysfunction including diabetes, neurodegenerative disease, traumatic brain injury, and acute organ injury (Hwang; Lifshitz et al., 2004; Yan et al., 2011; Pundik et al., 2012; Cheng and Ristow, 2013). Ischemia/reperfusion and drug/toxicant-induced renal injury demonstrates mitochondrial dysfunction and suppression of MB, and recovery of renal function is tightly linked to the restoration of mitochondrial number and function (Funk and Schnellmann, 2012). This suggests that development of therapies capable of inducing MB may have great potential in the treatment of a broad range of disease states.

Despite strong evidence supporting mitochondria as a therapeutic target, there are very few drugs/chemicals available that promote mitochondrial function or MB. Many of the agents that are available suffer from lack of specificity, low potency or poor toxicity profiles. There is a clinical need to develop new pharmacological agents or identify existing therapeutics that induces MB. Due to the role of cyclic nucleotides as regulators of PGC-1α, in this study, we sought to determine the efficacy of various classes of PDE inhibitors at stimulating MB.

The cAMP/PKA/CREB signaling cascade is a well-characterized regulator of PGC-1α expression and activity (Fernandez-Marcos and Auwerx, 2011). Increases in intracellular cAMP levels cause activation of protein kinase A (PKA), and subsequent phosphorylation and activation of CREB, an important transcriptional regulator of PGC-1α. Induction of cAMP levels in the cell occurs following activation of various G-protein coupled receptors. Our lab recently

identified the β2-adrenergic agonist, formoterol, as a potent inducer of MB in kidney and heart (Wills et al., 2012). β-agonism has previously been shown to induce PGC-1α in skeletal muscle of treated mice (Miura et al., 2007). Additionally, exercise-induced MB can be blocked by treatment with β-receptor antagonists, propranolol and ICI-118,551. cAMP levels in the cell are controlled both by the rate of synthesis and the rate of turnover by cyclic nucleotide phosphodiesterases. Therefore, inhibition of PDEs that hydrolyze cAMP may serve as a viable intervention to induce MB.

To test this hypothesis, we screened a panel of PDE3, PDE4 and PDE5 inhibitors using a phenotypic respirometric assay. FCCP-uncoupled OCR was used as a marker of increased energetic capacity and MB. Interestingly, PDE3 and PDE5 inhibitors increased FCCP-uncoupled OCR in RPTC, while none of the PDE4 inhibitors tested caused an increase (Fig. 1, 4). To further probe the functional selectivity of PDE3, PDE4 and PDE5 inhibition in promoting MB, cAMP and cGMP levels were measured in RPTC after treatment with the PDE3 inhibitors cilostamide and trequinsin, the PDE4 inhibitor rolipram or the PDE5 inhibitor sildenafil. PDE3 inhibition led to increases in levels of both cAMP and cGMP in RPTC, while rolipram increased only cAMP levels, and sildenafil increased only cGMP levels (Fig 3). These data correspond with the classical substrate affinities of the various PDE family members: PDE3 hydrolyzes both cAMP and cGMP with nearly equal affinity, PDE4 specifically hydrolyzes cAMP and PDE5 specifically hydrolyzes cGMP (Bender and Beavo, 2006; Francis et al., 2011). Finally, 8-Br-cGMP increased FCCP-uncoupled OCR in the respirometric assay and increased mRNA expression of mitochondrial genes after 24h treatment. 8-Br-cAMP had no effect on respiration of mitochondrial gene expression in RPTC. This multi-pronged approach strongly supports our hypothesis that cGMP, rather than cAMP is important for regulation of MB in renal tubules.

cGMP has previously been demonstrated to induce MB through the eNOS/NO/sGC/cGMP signaling cascade. Nisoli et. al. in 2004 showed that long term administration of NO mimetics,

guanylyl cyclase activators or 8-Br-cGMP increased mRNA expression of mitochondrial genes, mtDNA copy number, mitochondrial respiration and ATP levels in multiple cell lines (Nisoli et al., 2004). eNOS deficient mice have a reduction in metabolic rate and accelerated weight gain, which has been correlated with reduced mitochondrial content and function (Nisoli et al., 2003).

Both the PDE3 inhibitors cilostamide or trequinsin (0.3-3 mg/kg) and the PDE5 inhibitor sildenafil (0.3-3 mg/kg) when administered to naïve mice induced renal cortical mRNA expression of PGC-1α, nuclear encoded-mitochondrial genes (NDUFB8 and ATPSβ) and mitochondrial encoded-mitochondrial genes (ND1 and COX1). mtDNA copy number was also increased in the renal cortex of these mice (Fig 5, 6). Sildenafil increased the number of functional mitochondria in the renal cortex as evidenced by a significant increase in tissue ATP levels (Fig 6). These data confirm that PDE3 and PDE5 inhibitors are capable of inducing MB both in vitro in RPTC and in vivo in mouse kidney.

Cyclic nucleotides including both cAMP and cGMP have been demonstrated to be activators of signaling pathways promoting MB in various model systems (Nisoli et al., 2003; Nisoli et al., 2004; Tadaishi et al., 2011; Muller et al., 2013). Acute ex vivo administration of the PDE5 inhibitor vardenafil to human skeletal muscle stimulated MB as evidenced by increases in mitochondrial gene expression and mtDNA copy number (De Toni et al., 2011). This is the first report of pharmacological induction of MB in vivo by inhibition of either PDE3 or PDE5, and could represent a novel use for these classes of compounds. Despite the evidence of their role in MB, these compounds have yet to be evaluated as potential therapies for mitochondrial damage and dysfunction.

Previous studies reported the ability of various classes of phosphodiesterase inhibitors to protect against AKI. Pretreatment with rolipram, a specific PDE4 inhibitor, blunted I/R induced renal dysfunction in rat kidney and reduced oxidative damage (Mammadov et al., 2012).

Sildenafil was shown to be protective in cisplatin-induced AKI, while tadalafil, a long acting PDE5 inhibitor, protected against early I/R injury in rats (Lee et al., 2009; Sohotnik et al., 2013). However, limitations of these studies have been the lack of a clear mechanism for the renoprotective effects and the use of pre-treatment protocols. To address these issues, we examined the ability of sildenafil to promote recovery from FA-induced AKI by administering the drug 24h after induction of injury, and examined the effects of FA and sildenafil on both renal and mitochondrial function. Sildenafil promoted recovery mitochondrial gene expression (i.e. COX1 and Tfam) and mtDNA copy number. Additionally, renal KIM-1 expression was reduced in sildenafil treated mice indicating an enhanced recovery from the renal injury. These results demonstrate that sildenafil accelerates recovery from AKI by activating MB pathways.

Our results indicate that PDE inhibitors that are capable of increasing tissue levels of cGMP including sildenafil are promising treatments for diseases characterized by mitochondrial dysfunction and suppression of MB, including acute kidney injury.

Authorship Contributions

Participated in research design: Whitaker RM, Wills LP, Stallons LJ, and Schnellmann RG

Conducted experiments: Whitaker RM, Stallons LJ, and Wills LP

Performed data analysis: Whitaker RM

Wrote or contributed to the writing of the manuscript: Whitaker RM, and Schnellmann RG

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Footnotes

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Figure Legends

Figure 1: PDE3 inhibitors, but not PDE4 inhibitors increase FCCP-induced uncoupled mitochondrial respiration in RPTC. RPTC were treated with cilostamide (A), trequinsin (B), (R)-(-)-rolipram (C), or Ro 20-1724 (D) for 24 h. FCCP-uncoupled mitochondrial respiration was measured using the Seahorse XF-96 instrument. Data are represented as mean ± S.E.M., n≥3. *, $p < 0.05$ vs. vehicle control.

Figure 2: PDE3 inhibitors cilostamide or trequinsin induce mitochondrial protein gene expression in RPTC. RPTC were exposed to cilostamide (25 nM) or trequinsin (30 nM) for 24 h and evaluated for changes in mRNA expression of PGC-1α, ND6, and NDUFβ8 relative to DMSO controls. Data are represented as mean ± S.E.M, n≥4. *, $p < 0.05$ vs. vehicle control.

Figure 3: PDE inhibitor induced increases in cGMP, but not cAMP stimulate mitochondrial biogenesis in RPTC. cAMP (A) and cGMP (B) levels were measured in RPTC by ELISA 20 min after treatment with DMSO, cilostamide (25 nM), trequinsin (30 nM), rolipram (0.5 μM) or sildenafil (10 nM). FCCP-uncoupled mitochondrial respiration was measured using the Seahorse XF-96 instrument following 24h treatment with 8-Br-cAMP or 8-Br-cGMP (C). RPTC were exposed to 8-Br-cAMP (10 μM) or 8-Br-cGMP (10 μM) for 24 h and evaluated for changes in mRNA expression of PGC-1α, ND6, and NDUFβ8 relative to DMSO controls (D). Data are represented as mean ± S.E.M., n ≥ 3. *, $p < 0.05$ vs. vehicle control.

Figure 4: The PDE5 inhibitor sildenafil stimulates mitochondrial biogenesis in RPTC. Sildenafil increases FCCP-uncoupled mitochondrial respiration at various doses (A) and mitochondrial gene expression at 10 nM (B) in RPTC. mRNA expression of PGC-1α, ND6 and NDUFβ8 are represented as mean ± S.E.M. of at least three biological replicates. *, $P < 0.05$ vs. vehicle control.

Figure 5: PDE3 inhibitors cilostamide and trequinsin induce mitochondrial gene expression and mtDNA copy number in mouse renal cortex. mRNA expression and mtDNA copy number were evaluated in the renal cortex of mice 24 h after a single intraperitoneal injection of cilostamide (A, C), or trequinsin (B, D). Values indicate fold change relative to DMSO controls. Data are represented as mean ± S.E.M., n≥4. *, $p < 0.05$ vs. vehicle control.

Figure 6: Sildenafil induces mitochondrial gene expression, mtDNA copy number and ATP levels in mouse renal cortex. mRNA expression, mtDNA copy number and ATP levels were evaluated in the renal cortex of mice 24 h after a single intraperitoneal injection of sildenafil (A, B, C). Values indicate fold change relative to DMSO controls. Data are represented as mean ± S.E.M., n≥4. *, $p < 0.05$ vs. vehicle control.

Figure 7: Sildenafil stimulates MB after FA-induced AKI. AKI was induced in C57BL/6 by a single intraperitoneal injection of FA. Mice received daily injections of sildenafil (0.3 mg/kg) or saline vehicle beginning 24h after FA. Mice were sacrificed and kidneys were collected 7d after FA administration. mRNA expression (A) and mtDNA copy number (B) were evaluated in the renal cortex. Immunoblotting was performed for renal cortical assessment of KIM-1 expression and quantified via densitometry (C, D). Data are represented as mean ± S.E.M. and $N \geq 3$.
*, $P < 0.05$ vs. vehicle control, #, $P < 0.05$ vs. FA.

Figure 1

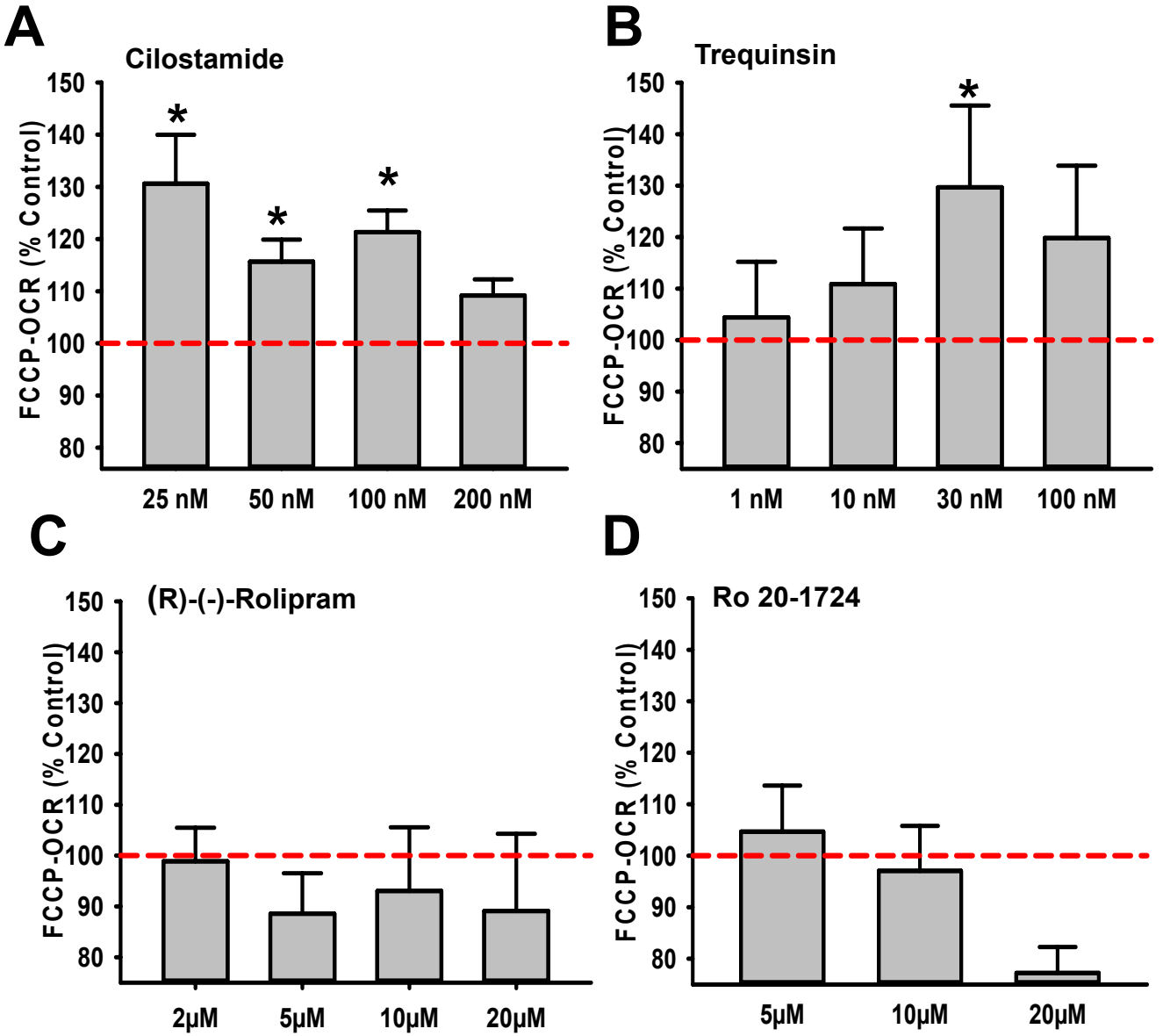

A
Cilostamide
FCCP-OCR (% Control)
150
140
130
120
110
100
90
80
*
*
*
25 nM
50 nM
100 nM
200 nM
B
Trequinsin
FCCP-OCR (% Control)
150
140
130
120
110
100
90
80
*
1 nM
10 nM
30 nM
100 nM
C
(R)-(-)-Rolipram
FCCP-OCR (% Control)
150
140
130
120
110
100
90
80
2μM
5μM
10μM
20μM
D
Ro 20-1724
FCCP-OCR (% Control)
150
140
130
120
110
100
90
80
5μM
10μM
20μM

Figure 2

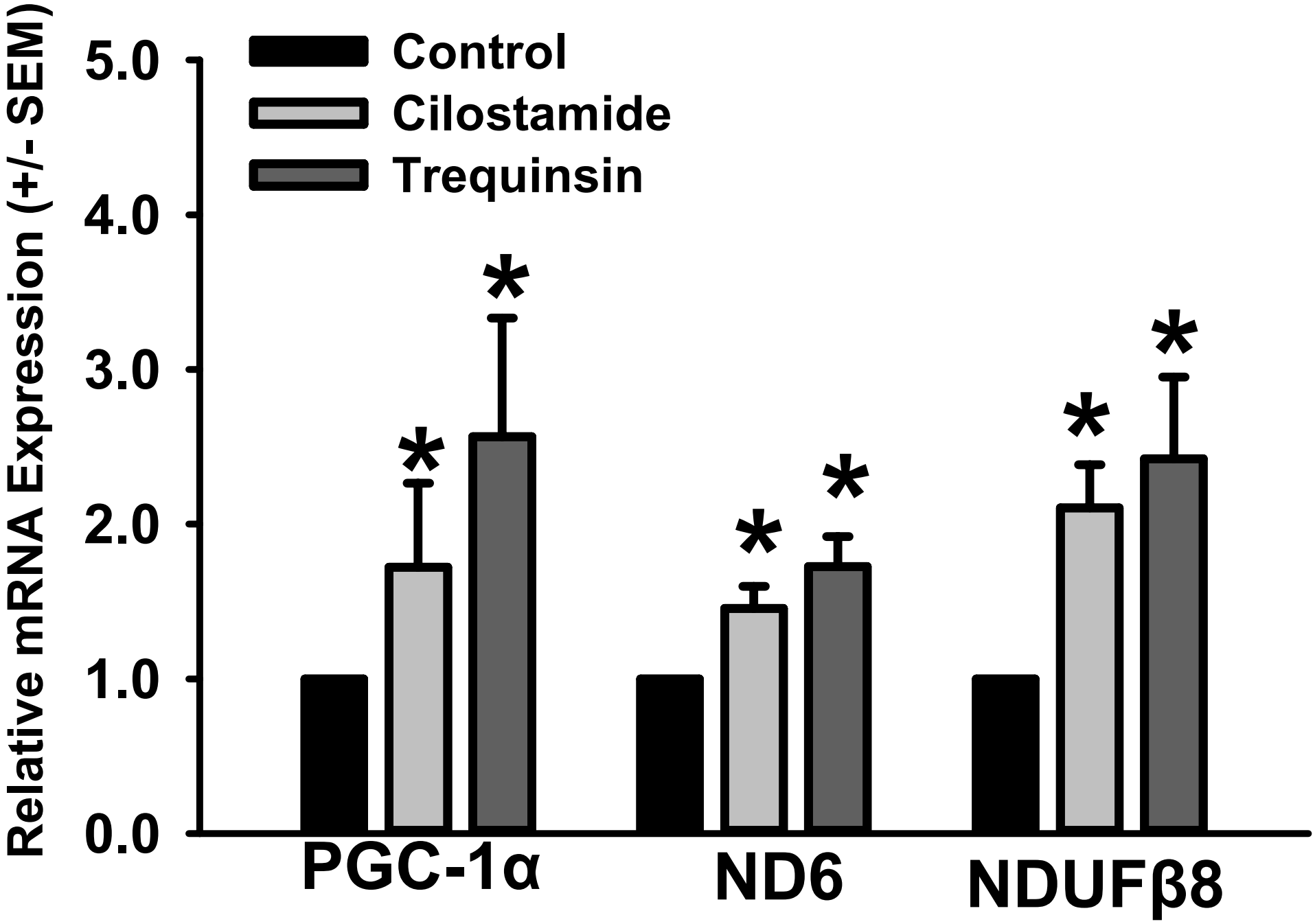

Control
Cilostamide
Trequinsin
Relative mRNA Expression (+/- SEM)
5.0
4.0
3.0
2.0
1.0
0.0
*
PGC-1α
ND6
NDUFβ8

Figure 3

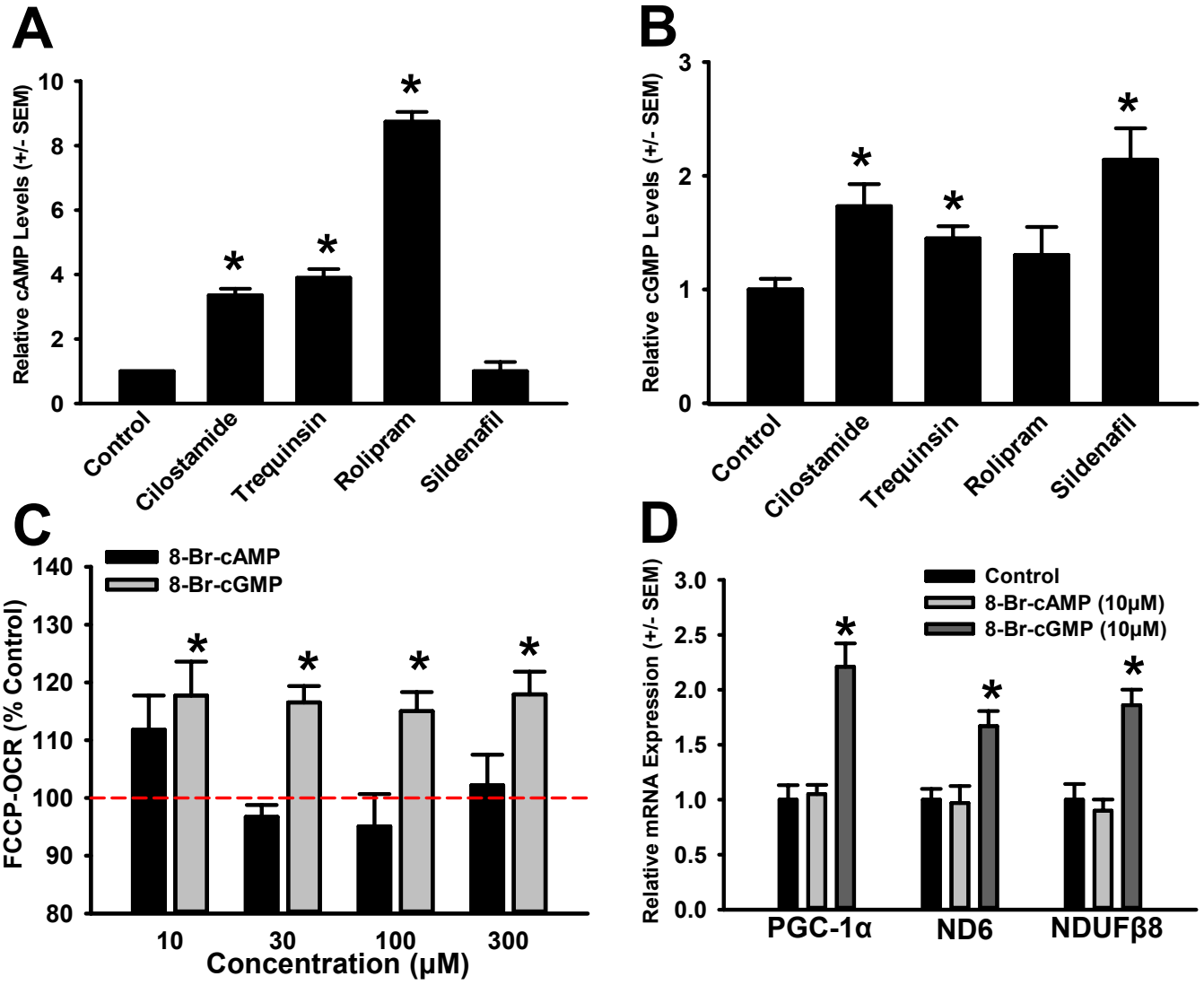

A
Relative cAMP Levels (+/- SEM)
10
8
6
4
2
0
*
*
*
Control
Cilostamide
Trequinsin
Rolipram
Sildenafil
B
Relative cGMP Levels (+/- SEM)
3
2
1
0
*
*
*
Control
Cilostamide
Trequinsin
Rolipram
Sildenafil
C
8-Br-cAMP
8-Br-cGMP
FCCP-OCR (% Control)
140
130
120
110
100
90
80
*
*
*
*
10
30
100
300
Concentration (μM)
D
Relative mRNA Expression (+/- SEM)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Control
8-Br-cAMP (10μM)
8-Br-cGMP (10μM)
*
*
*
PGC-1α
ND6
NDUFβ8

Figure 4

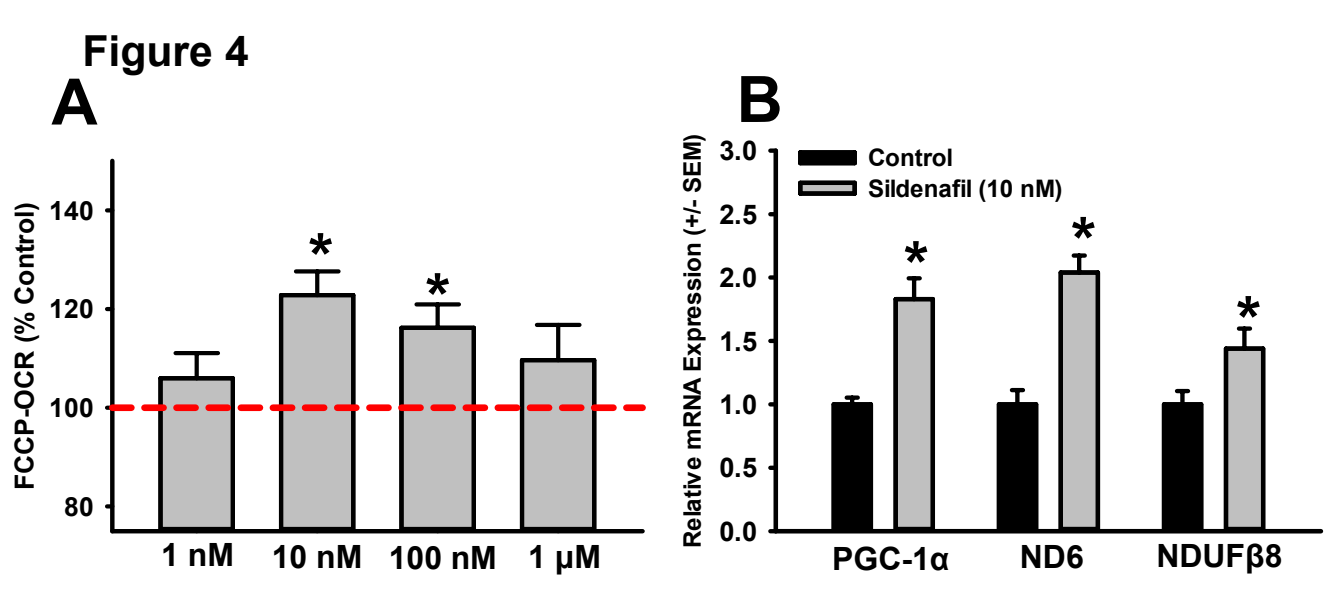
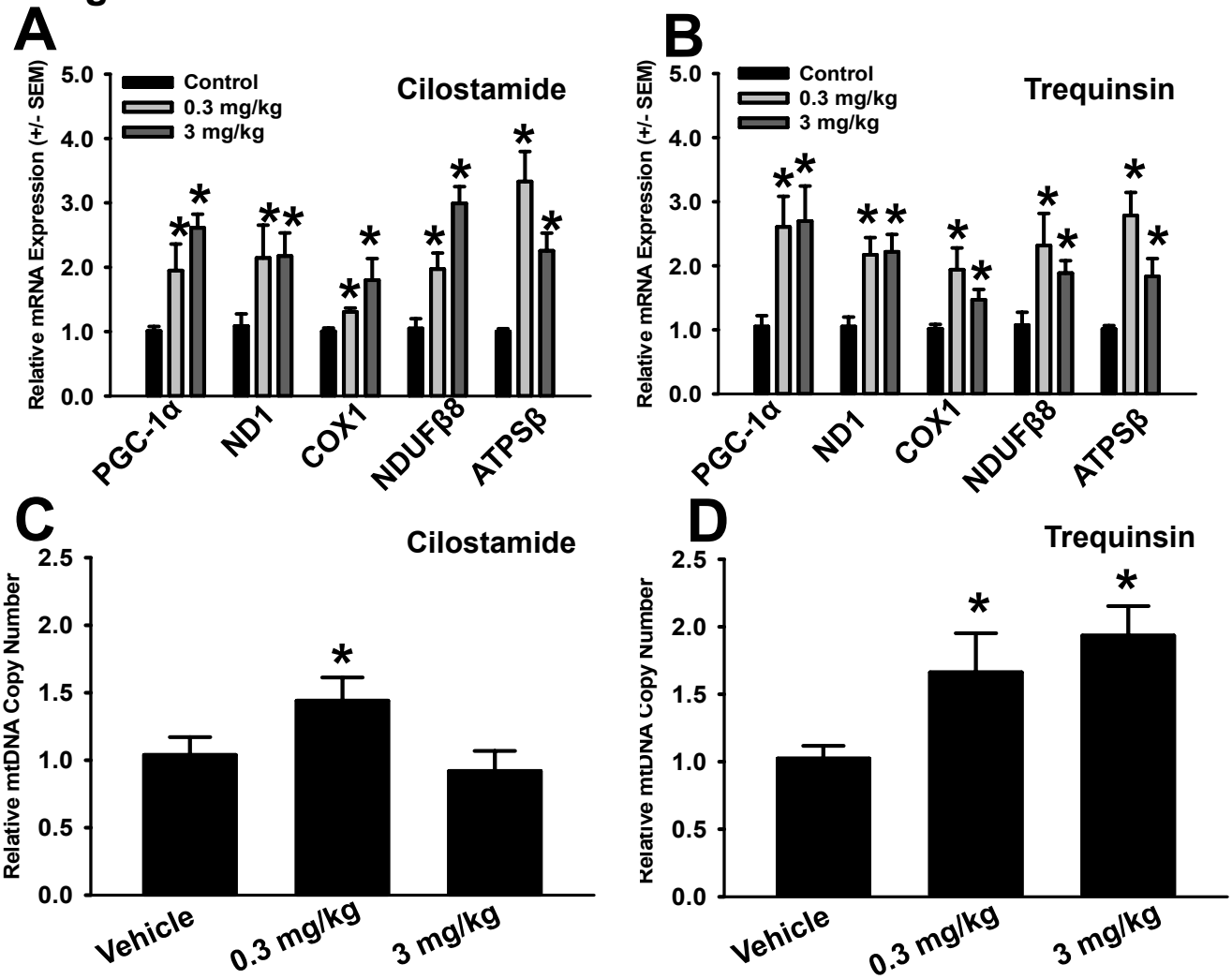
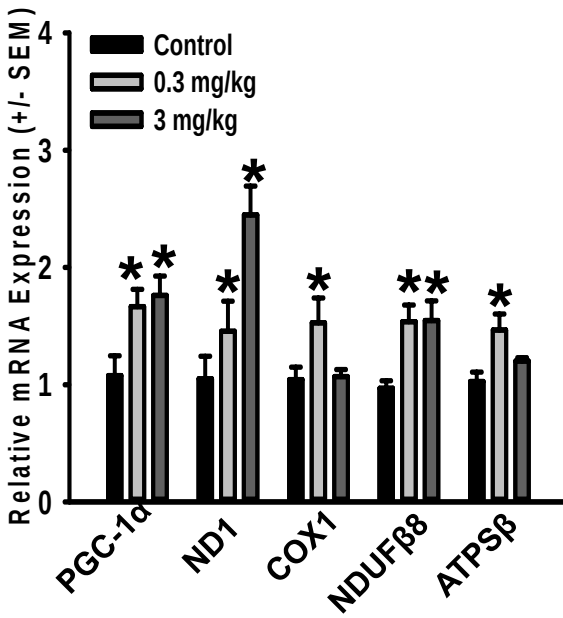

A
FCCP-OCR (% Control)
140
120
100
80
*
*
1 nM
10 nM
100 nM
1 µM
B
Relative mRNA Expression (+/- SEM)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Control
Sildenafil (10 nM)
*
*
*
PGC-1α
ND6
NDUFβ8

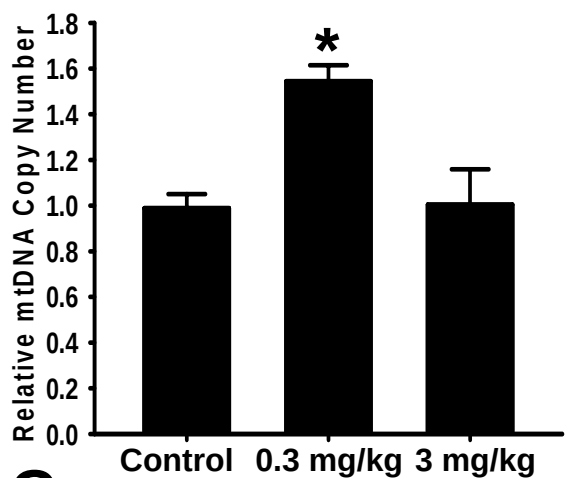
Figure 5


A
Cilostamide
Control
0.3 mg/kg
3 mg/kg
Relative mRNA Expression (+/- SEM)
5.0
4.0
3.0
2.0
1.0
0.0
PGC-1α
ND1
COX1
NDUFβ8
ATPSβ
B
Trequinsin
Control
0.3 mg/kg
3 mg/kg
Relative mRNA Expression (+/- SEM)
PGC-1α
ND1
COX1
NDUFβ8
ATPSβ
C
Cilostamide
Relative mtDNA Copy Number
2.5
2.0
1.5
1.0
0.5
0.0
Vehicle
0.3 mg/kg
3 mg/kg
D
Trequinsin
Relative mtDNA Copy Number
Vehicle
0.3 mg/kg
3 mg/kg

A Figure 6


Control
0.3 mg/kg
3 mg/kg
Relative mRNA Expression (+/- SEM)
0
1
2
3
4
PGC-1α
ND1
COX1
NDUFβ8
ATPSβ

B


Relative mtDNA Copy Number
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
Control
0.3 mg/kg
3 mg/kg

C

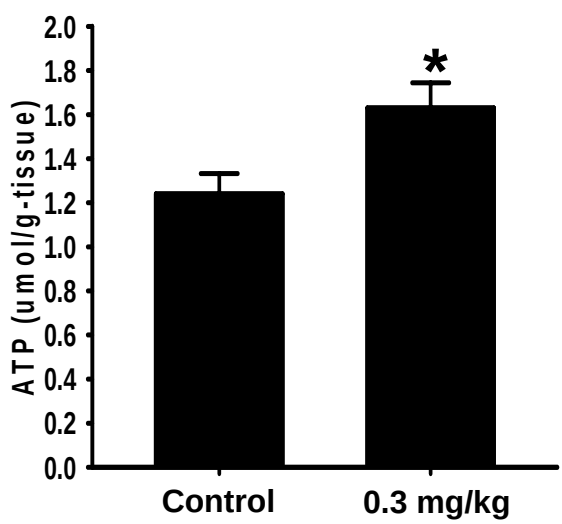
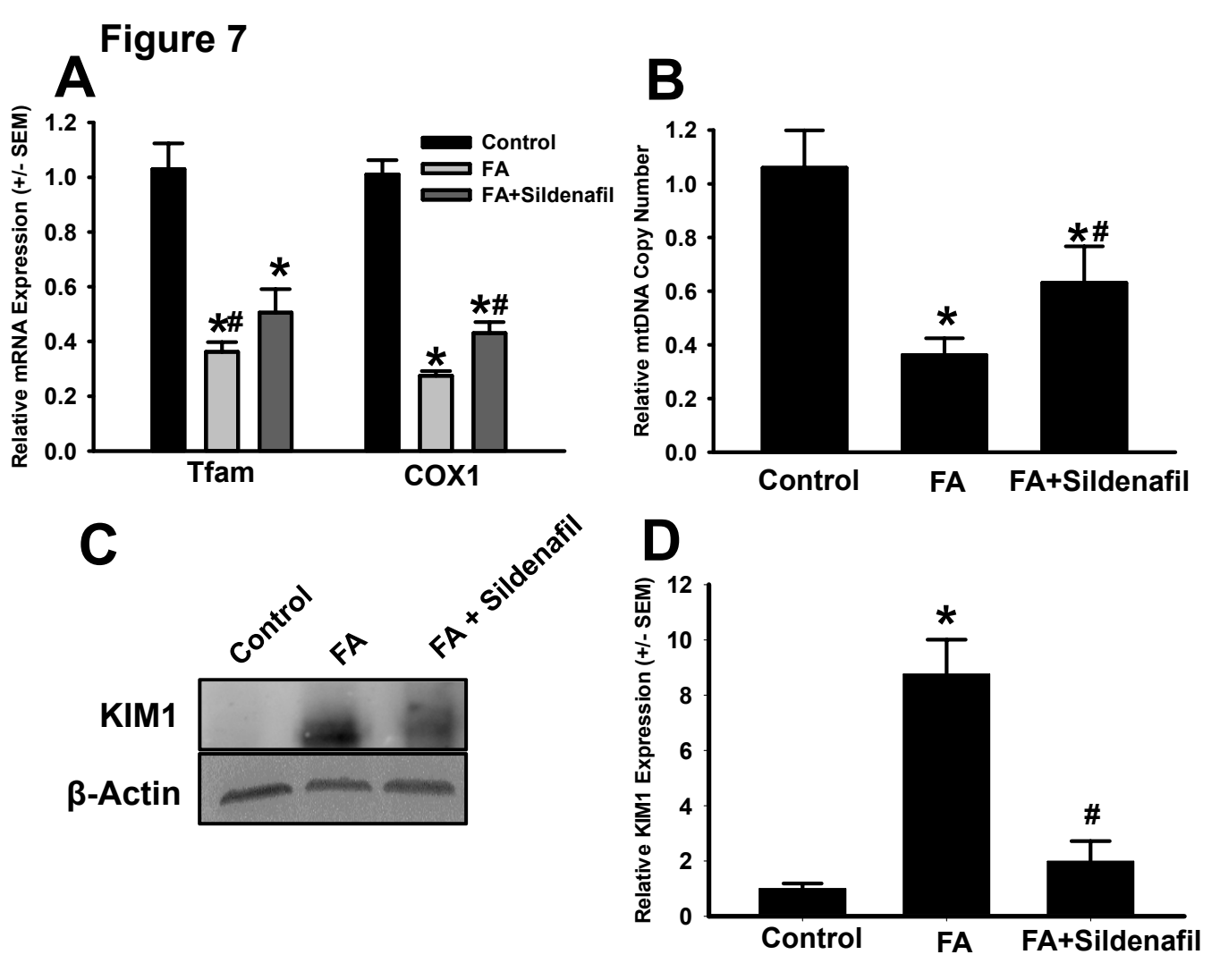

ATP (umol/g-tissue)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
Control
0.3 mg/kg

Figure 7


A
Relative mRNA Expression (+/- SEM)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control
FA
FA+Sildenafil
*#
*
*
*#
Tfam
COX1
B
Relative mtDNA Copy Number
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*#
Control
FA
FA+Sildenafil
C
Control
FA
FA + Sildenafil
KIM1
β-Actin
D
Relative KIM1 Expression (+/- SEM)
12
10
8
6
4
2
0
*
#
Control
FA
FA+Sildenafil