Title

Agonism of the 5-Hydroxytryptamine 1F Receptor Promotes Mitochondrial Biogenesis and Recovery from Acute Kidney Injury

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Running Title

5-HT_{1F} receptor agonism promotes mitochondrial biogenesis

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5-HT, 5-hydroxytryptamine, serotonin; αm5-HT, α-methyl 5-hydroxytryptamine; AKI, acute kidney injury; ANOVA, analysis of variance; ATP, adenosine triphosphate; ATP Synth, ATP synthase β; BUN, blood urea nitrogen; Cox1, cytochrome c oxidase 1; Ct, threshold cycle; DOI, 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane; DMSO, dimethyl sulfoxide; FCCP, carbonylcyanide-p-trifluoromethoxyphenylhydrazone; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HT, heart; I/R, ischemia/reperfusion; KIM-1, kidney injury molecule 1; LV, liver; LY334370, 4-fluoro-N-[3-(1-methyl-4-piperidinyl)-1H-indol-5-yl]benzamide; LY344864, N-[(3R)-3-(dimethylamino)-2,3,4,9-tetrahydro-1H-carbazol-6-yl]-4-fluorobenzamide; MB, mitochondrial biogenesis; mtDNA, mitochondrial DNA; NDUFB8, NADH dehydrogenase (ubiquinone) 1β subcomplex subunit 8; OCR, oxygen consumption rate; OXPHOS, oxidative phosphorylation; PGC1α, peroxisome proliferator-activated receptor coactivator 1-alpha; PPAR, peroxisome proliferator-activated receptor; qPCR, quantitative polymerase chain reaction; RPTC, renal proximal tubule cells; siRNA, small interfering ribonucleic acid

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Abstract

Many acute and chronic conditions, such as acute kidney injury, chronic kidney disease, heart failure, and liver disease involve mitochondrial dysfunction. While we have provided evidence that drug-induced stimulation of mitochondrial biogenesis (MB) accelerates mitochondrial and cellular repair leading to recovery of organ function, only a limited number of chemicals have been identified that induce MB. The goal of this study was to assess the role of the 5-HT1F receptor in MB. Immunoblot and qPCR analyses revealed 5-HT1F receptor expression in renal proximal tubule cells (RPTC). A MB screening assay demonstrated that two selective 5-HT1F receptor agonists, 4-fluoro-N-[3-(1-methyl-4-piperidinyl)-1H-indol-5-yl]benzamide (LY334370) and N-[(3R)-3-(dimethylamino)-2,3,4,9-tetrahydro-1H-carbazol-6-yl]-4-fluorobenzamide (LY344864, 1 – 100 nM) increased carbonylcyanide-p-trifluoromethoxyphenylhydrazone (FCCP)-uncoupled oxygen consumption in RPTC and validation studies confirmed both agonists increased mitochondrial proteins (e.g. ATP synthase β, Cox1, and NDUFB8) in vitro. siRNA knockdown of the 5-HT1F receptor blocked agonist-induced MB. Furthermore, LY344864 increased PGC1α, Cox1, and NDUFB8 transcript levels and mitochondrial DNA (mtDNA) copy number in murine renal cortex, heart, and liver. Finally, LY344864 accelerated recovery of renal function, as indicated by decreased blood urea nitrogen and KIM-1, and increased mtDNA copy number following ischemia/reperfusion-induced acute kidney injury (AKI). In summary, these studies reveal that the 5-HT1F receptor is linked to MB, 5-HT1F receptor agonism promotes MB in vitro and in vivo, and 5-HT1F receptor agonism promotes recovery from AKI injury. Induction of MB through 5-HT1F
receptor agonism represents a new target and approach to treat mitochondrial organ dysfunction.
1. Introduction

Mitochondrial dysfunction is linked to diverse acute insults such as surgery, trauma, ischemia/reperfusion (I/R) and drug toxicity, as well as chronic conditions such as metabolic syndrome, diabetes, neurodegenerative diseases, and aging (Bayeva, Gheorghiade, & Ardehali, 2013; Legrand, Mik, Johannes, Payen, & Ince, 2008; Monsalve, Borniquel, Valle, & Lamas, 2007; Pessayre et al., 2012; Robertson, 2004). Within the body, the organs most affected by mitochondrial dysfunction include those with the highest energy demand that primarily rely on mitochondrial aerobic respiration for ATP generation (e.g. kidney, heart, and brain) (Beeson, Beeson, & Schnellmann, 2010). Decreased mitochondrial function may not only cause cell death, but decrease cellular functions and reduce the ability of a cell to respond to increased cellular energy demand.

Induction of mitochondrial biogenesis (MB) has been shown to alleviate mitochondrial dysfunction following injury in several pathophysiological model systems (Dam, Mitchell, & Quadrilatero, 2013; Funk, Odejinmi, & Schnellmann, 2010; Funk & Schnellmann, 2013; Whitaker, Wills, Stallons, & Schnellmann, 2013). Requiring coordination of both the nuclear and mitochondrial genomes, MB is the synthesis of new mitochondria which is mediated by the master regulator peroxisome proliferator-activated receptor (PPAR) coactivator 1-alpha (PGC1α) (Scarpulla, Vega, & Kelly, 2012) and replaces defective or deficient mitochondria and/or supplements existing mitochondria to increase respiratory capacity. MB is an integral component of mitochondrial homeostasis that also includes mitophagy, fission, and fusion.
We have proposed that MB is a good drug target for diseases with mitochondrial dysfunction (Funk et al., 2010). Unfortunately, very few non-toxic chemicals or drugs suitable for pharmacological development have been identified that promote MB. For example, resveratrol (Csizsar et al., 2009; Mudo et al., 2012); catecholamines (Frier, Wan, Williams, Stefanson, & Wright, 2012); AMP-activated protein kinase activators like AICAR (Kukidome et al., 2006); the thiazolidinedione class of anti-diabetic drugs including rosiglitazone and pioglitazone (Pagel-Langenickel et al., 2008; Yan-Fei, Li-Hua, Xi, & Zhi-Wei, 2008); pyrroloquinoline quinine moieties of quinoproteins (Chowanadisai et al., 2010); and the dietary supplement beta-guanidinopropionic acid (Williams et al., 2009) have been shown to upregulate PGC1α. Mixed results have been obtained with the fibrate drug bezafibrate, a pan-PPAR agonist (Romanino et al., 2011; Wenz, Diaz, Spiegelman, & Moraes, 2008; Yatsuga & Suomalainen, 2012). A recent high-throughput screen in murine primary skeletal muscle cells elucidated several distinct classes of drugs promoting MB; however, several of these including glucocorticoids and inhibitors of microtubules and protein synthesis would not be suitable for clinical administration due to toxicity and side effects (Arany et al., 2008).

We previously identified isoflavones and isoflavone derivatives as promoters of MB, characterized the minimal pharmacophore, determined the mechanism of action, and demonstrated that isoflavone treatment following oxidant exposure accelerated recovery of mitochondrial and cellular function (Rasbach & Schnellmann, 2008). Additionally, the reported SIRT1 activator N-[2-[3-(piperazin-1-ylmethyl)imidazo[2,1-b][1,3]thiazol-6-yl]phenyl]quinoxaline-2-carboxamide (SRT-1720) promoted MB and expedited recovery of mitochondria following oxidant injury (Funk et al., 2010). More recently, β2-adrenergic
receptor agonists, such as formoterol, were also identified as potent promoters of MB in vitro and in vivo and a pharmacophore was developed (Peterson et al., 2013; Wills et al., 2012). Interestingly, not all β2 adrenoceptor agonists were capable of inducing MB (Peterson et al., 2013). Finally, specific inhibition of phosphodiesterases 3 and 5 increased cGMP to produce MB in renal proximal tubule cells (RPTC) and mice, and accelerated the recovery of renal function following acute kidney injury (Whitaker et al., 2013).

As part of our drug discovery program in MB, we explored the agonism of 5-hydroxytryptamine (5-HT, serotonin) receptors in MB. Agonism of the 5-HT2 receptor with 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI, 1 - 10 μM) promoted MB in vitro as evidenced by increased FCCP-uncoupled respiration, ATP, PGC1α promoter activity, and the mitochondrial proteins ATP synthase β and NDUFB8 [27]. Additionally, DOI accelerated restoration of mitochondrial function following oxidant injury (Rasbach, Funk, Jayavelu, Green, & Schnellmann, 2010). Considering that micromolar concentrations of DOI were needed to promote MB in vitro and that micromolar concentrations of DOI also activate the 5-HT1F receptor, we examined the role of the 5-HT1F receptor in MB.
2. Materials and Methods

2.1 In vitro Studies

Renal proximal tubules were isolated from kidneys of female New Zealand white rabbits (1.5 – 2 kg) using an iron oxide perfusion method and cultured under improved conditions resulting in normal aerobic metabolism comparable to that found in vivo (Nowak & Schnellmann, 1995). We have previously developed a high-throughput screening assay which identifies compounds that exhibit elevated carbonylcyanide-p-trifluoromethoxyphénylhydrzone (FCCP)-induced uncoupled oxygen consumption rates (OCR), indicative of increased mitochondrial capacity (Beeson et al., 2010). OCR were measured using a Seahorse Biosciences analyzer before (basal) and after addition of 1 μM FCCP, an ionophore that uncouples electron transport from ATP generation. The selective 5-HT$_{1F}$ receptor agonists 4-fluoro-N-[3-(1-methyl-4-piperidinyl)-1H-indol-5-yl]benzamide (LY334370) and N-[(3R)-3-(dimethylamino)-2,3,4,9-tetrahydro-1H-carbazol-6-yl]-4-fluorobenzamide (LY344864), and the non-selective 5-HT receptor agonist α-methyl 5-HT (αm5-HT) were purchased from Tocris (Ellisville, MO).

For 5-HT$_{1F}$ receptor knockdown, RPTC were treated with scrambled siRNA (siGENOME non-targeting siRNA#5, Dharmacon RNAi Technologies, Lafayette, CO) or siRNA designed against the 5-HT$_{1F}$ receptor using RNAiFect transfection reagent (Qiagen, Valencia, CA). siRNA knockdown was accomplished using a 1:1 mixture of siRNA recognizing the following sequences: siRNA-1, 5’-CCT TCA GCA TTG TGT ATA
T-3’ and siRNA-2, 5’- CCA CAT TGT TTC CAC TAT T-3’. Following a 72 hr exposure, cells were scraped and analyzed for changes in protein levels.

2.2 In vivo Studies

Non-fasted naïve eight- to ten-week-old male C57BL/6NCr mice (National Cancer Institute, Frederick, MD) weighing 25-30 g received an intraperitoneal injection of 250 μL vehicle (0.9% saline) or test compound every 8 hr for a total of three doses in a 24 hr period (1 mg/kg x 3) or one dose of test compound (2 mg/kg or 10 mg/kg). At time of euthanasia, organs were harvested, snap-frozen in liquid nitrogen, and stored at -80°C. For the I/R model of AKI, mice were subjected to bilateral renal pedicle ligation as described previously (Jesinkey et al., 2014; Zhuang et al., 2009). Briefly, renal artery and vein were isolated and blood flow was occluded with a vascular clamp for 18 min. Mice were maintained at 37°C throughout the procedure using a homeothermic heating system. Sham surgery mice received all manipulations with the exception of clamping of the renal pedicles. Mice were treated once daily beginning at 24 hr after reperfusion with saline vehicle or LY344864 (2 mg/kg). Blood was collected from mice via retro-orbital bleed at 24 hr and 144 hr after surgery. Blood urea nitrogen (BUN) levels were measured using a QuantiChrom Urea Assay Kit (BioAssay Systems, Hayward, CA) according to the manufacturer’s protocol. Mice were euthanized at 144 hr after the procedure, at which time kidneys were harvested for molecular analyses. Animal protocol was approved by and procedures completed in compliance with IUCAC guidelines.
2.3 Nucleic Acid Isolation and qPCR

RPTC were scraped in TRIzol® and RNA was isolated using a phenol-based centrifugation method. DNA was isolated using a DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA). cDNA was reverse transcribed from 2 μg RNA using RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Waltham, MA), diluted 1:5, and 5 μL added to a real-time Maxima SYBR green qPCR master mix containing ROX (Thermo Fisher Scientific, Waltham, MA). The following primers were used: Actin forward 5’GGG ATG TTT GCT CCA ACC AA 3’, Actin reverse 5’GCG CTT TTG ACT CAA GGA TTT AA 3’; ApoB forward 5’ CGT GGG CTC CAG CAT TCT 3’, ApoB reverse 5’ TCA CCA GTC ATT TCT GCC TTT G 3’; ATP Synthase β forward 5’ GAG ACC AAG AAG GTC AAG ATG 3’, ATP Synthase β reverse 5’ GAA GGG ATT CGG CCC AAT AAT GCA G 3’; Cox1 forward 5’ TAA TGT AAT CGT CAC CGC ACA 3’, Cox1 reverse 5’ ATG TGA GGA GCC CCA ATT ATC 3’; D Loop forward 5’ CCCAAG CAT ATA AGC TAG TA 3’, D Loop reverse 5’ ATA TAA GTC ATA TTT TGG GAA CTA C 3’; NDUFB8 forward 5’ ACC CAA TCC AAC CGC CTT CA 3’, NDUFB8 reverse 5’ CTA GGA CCC CAG AGG AAC GC 3’; PGC1α forward 5’ AGG AAA TCC GAG CTG AGC TGA ACA 3’, and PGC1α reverse 5’ GCA AGA AGG CGA CAC ATC GAA CAA 3’. Changes in gene expression were calculated based on the delta-delta Ct method. Mitochondrial DNA (mtDNA) copy number was calculated based on comparison of mitochondrial D Loop to nuclear ApoB.
2.4 Protein Isolation and Western Blotting

Freshly isolated renal proximal tubules or RPTC (cultured until confluent, about 6 days) were rinsed with ice-cold phosphate buffered saline, pelleted, and subjected to membrane fractionation (Subcellular Protein Fractionation Kit, Pierce Biotechnology, Rockford, IL). For non-fractionated samples, RPTC were scraped in radioimmuno-precipitation assay buffer containing protease inhibitors and phosphatase inhibitors (Sigma-Aldrich, St. Louis, MO). Following sonication, protein was quantified using a bicinchoninic acid assay, subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred onto nitrocellulose membranes, and incubated with primary and secondary antibodies (GAPDH from Fitzgerald, Acton, MA; Cox1 and NDUFB8 from Invitrogen, Frederick, MD; Kim-1 from R&D Systems Inc., Minneapolis, MN; ATP synthase β, 5-HT₁F receptor, rabbit and mouse secondary antibodies from AbCam, Cambridge, MA). Images were acquired with AlphaEase software (Protein Simple, Santa Clara, CA) and processed using ImageJ software.

2.5 Statistics

One way analysis of variance (ANOVA) or Student’s t test was used, as appropriate, to analyze data for significance (p < 0.05). Significance in ANOVA was scrutinized for multiple comparisons using the Fisher least significant difference post-hoc test. When normality failed, a one-way ANOVA on Ranks was performed.
3. Results

3.1 The 5-HT_{1F} receptor is present in RPTC and 5-HT_{1F} receptor agonism leads to an increase in FCCP-uncoupled OCR

To verify the expression of the 5-HT_{1F} receptor in our model system, RNA and protein were isolated from renal proximal tubules and RPTC. The 5-HT_{1F} receptor mRNA (362 bp) was observed in RPTC (Figure 1A) and the 5-HT_{1F} receptor protein (44 kDa) was present in freshly isolated renal tubules, tubule membrane fraction, RPTC, and RPTC membrane fraction (Figure 1B).

Previous studies have shown that LY334370 and LY344864 are specific agonists exhibiting high affinity for the 5-HT_{1F} receptor (Andrade et al., 2012; Ramadan, Skljarevski, Phebus, & Johnson, 2003). LY334370 and LY344864 increased FCCP-uncoupled OCR, a screening assay readout for MB [6], 1.2 - 1.4 fold and 1.2 - 1.5 fold, respectively, between 1 nM and 100 nM, but not at lower (0.1nM, 0.3nM) or higher (1 μM, 10μM) concentrations compared to control cells treated with vehicle (<0.5% DMSO) in RPTC after a 24 hr exposure (Figure 1C). Comparatively, the non-selective 5-HT receptor agonist αm5-HT increased FCCP-OCR 1.2 - 1.3 fold relative to control cells in RPTC at 24 hr. These results demonstrate that the 5-HT_{1F} receptor is present in RPTC and that the 5-HT_{1F} receptor agonists LY334370 and LY344864 increase FCCP-uncoupled OCR.

3.2 5-HT_{1F} receptor agonists increase mitochondrial proteins in RPTC

MB increases the quantity of electron transport chain proteins and copies of mtDNA, requiring coordination of both the nuclear and mitochondrial genomes. Three
representative oxidative phosphorylation (OXPHOS) proteins were measured to confirm MB: ATP synthase β (ATP Synth), a portion of the F₀-F₁ ATP synthase enzyme (nuclear-encoded); cytochrome c oxidase 1 (Cox1), a constituent of Complex IV (mitochondrial-encoded); and NADH dehydrogenase (ubiquinone) 1β subcomplex subunit 8 (NDUFB8), a nuclear-encoded component of Complex 1. The 5-HT₁F receptor agonist LY334370 increased OXPHOS protein expression 1.4-1.6 fold at 1 – 100 nM in RPTC at 24 hr (Figure 2A). Likewise, LY344864 (1 - 100 nM) similarly increased OXPHOS protein expression 1.4 - 2.1 fold in RPTC at 24 hr (Figure 2B). Thus, the 5-HT₁F receptor agonists LY334370 and LY344864 increased the levels of ATP Synth, Cox1, and NDUFB8 proteins at concentrations that also increased uncoupled respiration, consistent with MB.

3.3 siRNA knockdown of the 5-HT₁F receptor blocks MB in RPTC

To confirm that the MB actions of LY334370 and LY344864 were mediated through the 5-HT₁F receptor, siRNA transfection was used to knockdown 5-HT₁F receptor protein expression. Transfection of RPTC with siRNA targeted against the 5-HT₁F receptor decreased 5-HT₁F receptor protein 38% after 72 hr compared to scramble-treated RPTC (Figure 3A). Interestingly, 5-HT₁F receptor knockdown alone led to a significant diminution in ATP synth, Cox1, and NDUFB8 protein levels (Figure 3B). Knockdown of the 5-HT₁F receptor protein blocked LY334370- and LY344864-induced upregulation of Cox1 and NDUFB8 proteins (Figure 3C). These data reveal that the 5-HT₁F receptor may regulate MB under basal conditions and that post-transcriptional silencing of the 5-HT₁F receptor blocked the agonist-stimulated MB response.
3.4 5-HT_{1F} receptor agonism increases OXPHOS gene expression and mitochondrial DNA copy number in renal cortex

A published study showed that rat brain cortex levels of LY344864 remained constant for 6 hr while plasma levels declined over time following an intravenous dose of 1 mg/kg LY344864 (Phebus et al., 1997). Thus, an *in vivo* time course experiment (1, 8, 24 hr) was performed to determine gene expression and mtDNA copy number changes in renal cortex induced by vehicle (0.9% saline) or LY344864 (2 mg/kg, intraperitoneal) (Figure 4A). PGC1α and Cox1 mRNA were significantly increased at 1 hr and 8 hr but not at 24 hr. NDUFB8 gene expression was unchanged after 1 hr, but was upregulated 8 - 24 hr. Though mtDNA copy number was unchanged at 1 - 8 hr, it was increased at 24 hr. These results indicate promotion of MB through agonism of the 5-HT_{1F} receptor *in vivo*.

Subsequent to the initial time course experiment, multiple dosing regimens were assessed. Mice were injected intraperitoneally with vehicle (0.9% saline), 1 mg/kg LY344864 every 8 hr for a total of 3 doses in a 24 hr period (1 mg/kg x 3), one bolus dose of 2 mg/kg LY344864, or one bolus dose of 10 mg/kg LY344864, and sacrificed at 24 hr (Figure 4B). Gene expression of PGC1α and Cox1 in the renal cortex was significantly upregulated at 24 hr after 1 mg/kg x 3, unchanged with 2 mg/kg, and decreased after 10 mg/kg LY344864 compared to vehicle. NDUFB8 mRNA was increased with 1 mg/kg x 3 and 2 mg/kg but not 10 mg/kg LY344864 at 24 hr. Finally, mtDNA copy number was unchanged after 1 mg/kg x 3 and increased after 2 mg/kg and 10 mg/kg LY344864 at 24 hr in renal cortex. These data reveal that agonism of 5-HT_{1F} receptor promotes MB *in vivo* in the kidney.
3.5 The 5-HT$_{1F}$ receptor is present in murine cardiac and hepatic tissues and 5-HT$_{1F}$ receptor agonism increases OXPHOS expression and mitochondrial DNA copy number

To determine whether 5-HT$_{1F}$ receptor-mediated MB was selective for the kidney or also stimulated MB in other tissues, OXPHOS gene expression and mtDNA copy number were evaluated in the heart and the liver. 5-HT$_{1F}$ receptor mRNA (362 bp) was observed in both cardiac and hepatic tissues (Figure 5A). In the heart, LY344864 (2 mg/kg) increased PGC1$\alpha$ mRNA at 1 - 8 hr but not at 24 hr (Figure 5B). Cox1 gene expression was unchanged at 1 hr, but was upregulated 8 - 24 hr. NDUFB8 mRNA was increased at 8 hr but not at 1 hr or 24 hr. Though unchanged at 1 - 8 hr, mtDNA copy number increased with LY344864 treatment at 24 hr in the heart. In the liver, PGC1$\alpha$, Cox1, and NDUFB8 gene expression was significantly upregulated at 8 - 24 hr but not at 1 hr (Figure 5C). Hepatic tissue mtDNA copy number was unchanged at 1 - 8 hr but was significantly increased at 24 hr. Taken together, these data indicate that LY344864 promotes MB through upregulation of PGC1$\alpha$ and other OXPHOS genes culminating in an increase in mtDNA copy number in extra-renal heart and liver tissues within 24 hr.

3.6 LY344864 increases mitochondrial DNA copy number and promotes recovery from ischemia/reperfusion-induced acute kidney injury

Stimulation of MB has been previously reported to accelerate the recovery of renal structure and function after AKI (Jesinkey et al., 2014; Whitaker et al., 2013). We examined the ability of LY344864 to stimulate MB and promote renal recovery in an I/R-induced AKI model. Mice were subjected to bilateral renal ischemia and treated daily with saline vehicle or LY344864 (2 mg/kg) over the course of 144 hr following surgery.
All I/R-AKI mice had equal initial injury (BUN levels of 93 ± 15 mg/dL at 24h), but vehicle-treated mice failed to recover normal renal function as demonstrated by persistently elevated BUN levels (75 ± 6 mg/dL) (Figure 6A). Mice treated with LY344864 showed accelerated recovery of renal function as evidenced by a decrease in blood urea nitrogen (BUN) levels from initiation of treatment at 24 hr (99 ± 11 mg/dL) to near-control levels at the completion of treatment at 144 hr (45 ± 7 mg/dL).

To assess renal tubular recovery, renal cortical kidney injury molecule 1 (KIM-1) levels were measured by immunoblot analysis (Figure 6B). KIM-1 levels were upregulated in mice 144 hr after I/R injury compared to mice receiving no surgery or sham surgery. However, mice treated with LY344864 exhibited reduced KIM-1 protein expression in the renal cortex compared to vehicle-treated mice, demonstrating an accelerated recovery of the proximal tubular epithelium. Finally, the observed recovery of renal function was associated with recovery of mtDNA copy number. Renal cortical mtDNA copy number was 42% of sham surgery control levels at 144 hr after surgery in vehicle-treated I/R mice and treatment with LY344864 promoted the recovery of mtDNA copy number to 75% of sham surgery control levels (Figure 6C). These data provide strong evidence that 5-HT<sub>1F</sub> receptor agonism is a viable strategy to stimulate recovery of renal function in the setting of AKI, and that recovery is correlated with a restoration of mtDNA copy number and function.
4. Discussion

Historically, the 5-HT$_{1F}$ receptor has been reported to be a neuronal receptor in the central nervous system (CNS) mediating pain and lacking vasoactive properties, which has led to the development of 5-HT$_{1F}$ receptor agonists for the treatment of migraines (Mitsikostas & Tfelt-Hansen, 2012). However, despite its potential role in migraine pathogenesis, other physiological functions for 5-HT$_{1F}$ receptors have yet to be established.

In a high-throughput MB screening assay incorporating FCCP-OCR and the Seahorse Biosciences analyzer (Beeson et al., 2010), the selective 5-HT$_{1F}$ receptor agonists LY334370 and LY344864 potently induced uncoupled oxygen consumption in RPTC. Validation assays revealed increased levels of both mitochondrial-encoded (Cox1) and nuclear-encoded (ATP Synth and NDUFB8) proteins with 5-HT$_{1F}$ receptor agonist treatment, consistent with MB. Since 5-HT$_{1F}$ receptor antagonists have not been reported, siRNA was used to knockdown 5-HT$_{1F}$ receptors. A modest 38% knockdown of the 5-HT$_{1F}$ receptor not only blocked the MB effects of LY334370 and LY344864, but also significantly decreased basal levels of mitochondrial proteins in the kidney up to 50%. Time- and dose-dependent changes in OXPHOS genes and mitochondrial copy number were found in vivo in kidney, heart, and liver in response to 5-HT$_{1F}$ receptor agonism. This is the first report that 5-HT$_{1F}$ receptors are associated with mitochondrial function, including MB, and that agonists to this receptor promote MB in multiple tissues.
Both LY334370 and LY344864 have been reported to be selective and efficacious agonists at the 5-HT_{1F} receptor with reported pKd values of 8.7 and 8.2 for LY334370 and LY344864, respectively (Andrade et al., 2012; Ramadan et al., 2003). LY334370 also has affinity for the 5-HT_{1A} receptor (7.8 pKd) while LY344874 binds with 100 fold greater affinity at the 5-HT_{1F} receptor than other 5-HT_{1} receptors. Though LY334370 showed no overt adverse effects in our studies, preclinical toxicology studies led to identification of the liver as a potential target of injury in beagle dogs when administered for longer than one month (Ramadan et al., 2003). However, no toxicity was shown in rats and there was no increase in liver enzymes when administered to humans through Phase II clinical trials (Ramadan et al., 2003). There are no reports that LY344864 is toxic. Therefore, while both agonists performed equally in vitro, only LY344864 was utilized for in vivo studies in this report.

5-HT receptors are grouped into seven families of either ligand-gated ion channels (5-HT_{3} receptors) or class A (rhodopsin-like) guanine nucleotide-binding protein (G-protein)-coupled receptors (5-HT_{1,2,4-7} receptors), which are further divided into multiple subtypes. The majority of the 5-HT receptors are located in the CNS, platelets, and the gastrointestinal tract where they mediate diverse physiological processes, including anxiety, sleep, and appetite (Nichols & Nichols, 2008). The 5-HT_{1F} receptor shares 42 - 57% amino acid identity with the other 5-HT_{1} receptors (Barnes & Sharp, 1999), though its localization to various sites within the brain (e.g. cortex, hippocampus, and cerebellum), as well as outside of the central nervous system including reproductive tissues (uterus, testes), mesentery tissue, retina/whole eye, small intestine, kidney, liver, and heart (Bouchelet, Case, Olivier, & Hamel, 2000; Lovenberg et al., 1993;
Lucaites, Krushinski, Schaus, Audia, & Nelson, 2005; Su et al., 2004; Xu et al., 2007) suggests that agonism of the 5-HT$_{1F}$ receptor has the potential to promote MB across a wide range of tissues. The exact signaling mechanism mediating MB through 5-HT$_{1F}$ receptor agonism in RPTC still needs to be determined.

PGC1α mediates MB through activation of transcription factors such as the nuclear respiratory factors 1 & 2 and the estrogen-related receptors (ERR) in the nuclear genome; the NRFs trigger the nuclear-encoded mitochondrial transcription factor A (TFAM) which drives the mitochondrial gene transcription and genome replication, while the ERRs and other transcription factors activate transcription of nearly a thousand mitochondrial genes of nuclear origin (Ventura-Clapier, Garnier, & Veksler, 2008). In renal cortex, 5-HT$_{1F}$ receptor agonism led to a rapid induction of PGC1α (e.g. 1 hr) promoting MB, and resulting in an increase in mtDNA copy number within 24 hr. Timely responsiveness of PGC1α levels have also been reported after exercise and experimentally-induced sepsis, confirming the importance of bioenergetics in response to environmental stimuli (Mathai, Bonen, Benton, Robinson, & Graham, 2008; Sweeney, Suliman, Hollingsworth, & Piantadosi, 2010). Addition of 5-HT$_{1F}$ receptor agonist (1 mg/kg x 3) led to an increase in PGC1α, Cox1, and NDUFB8 transcript levels, but not a change in mtDNA copy number at 24 hr; these increased gene expression levels are most likely due to a more recent exposure to agonist (8 hr prior to sacrifice), and an incomplete MB program that perhaps takes longer than 24 hr to complete. A single dose of 2 mg/kg agonist led to a complete MB program in 24 hr as evidenced by early increases in PGC1α and OXPHOS expression followed by an increase in mtDNA copy number at 24 hr. Dosing at a higher concentration (10 mg/kg) also led to a complete
MB program with increased mtDNA copy number at 24 hr; however, down-regulation of MB gene expression (PGC1α, Cox1) at 24 hr presumes an earlier robust upregulation of the MB program with subsequent negative feedback inhibition occurring at 24 hr. Thus, activation of PGC1α precedes increases in gene expression of target OXPHOS genes, ultimately culminating in greater quantities of mtDNA and increased mtDNA copy number at the end of the MB program.

5-HT¹F receptor agonism also led to MB in extra-renal tissues. Within the liver, there was a robust induction of PGC1α and OXPHOS gene expression at 8 - 24 hr with an increase in mtDNA copy number at 24 hr. Promotion of MB was even more pronounced in the heart as 5-HT¹F receptor agonism led to a seven-fold increase in mtDNA copy number at 24 hr. Differences in the amplitude of the MB response among tissues indicate greater specificity of agonist at the tissue or greater 5-HT¹F receptor density/expression.

We have proposed that drugs inducing MB have the potential to improve therapeutic outcomes in the treatment of acute and chronic diseases in which mitochondrial dysfunction occurs (Funk et al., 2010). Mitochondrial dysfunction is commonly observed in acute organ injury of the kidney, heart, and liver (Di Lisa, Canton, Menabo, Kaludercic, & Bernardi, 2007; Havasi & Borkan, 2011; Jaeschke, McGill, & Ramachandran, 2012). Recovery of renal structure and function following AKI has been reported to be accelerated through stimulation of MB by agonism of the β₂-adrenergic receptor or by inhibition of cGMP-selective phosphodiesterases (Jesinkey et al., 2014; Whitaker et al., 2013). Likewise, stimulation of MB via agonism of the 5-HT¹F receptor with LY344864 herein was similarly able to promote accelerated renal recovery in an
I/R-induced AKI model. Thus, accelerated recovery of AKI, and possibly other acute or chronic diseases with mitochondrial dysfunction, can be induced through stimulation of MB through different upstream targets (e.g. 5-HT$_{1F}$ receptor agonism, β$_2$-adrenergic receptor agonism, phosphodiesterase inhibition), which is therapeutically beneficial due to differential patient diseases and responses. In addition to renal AKI, mitochondrial dysfunction is also involved in numerous neurodegenerative diseases, including Alzheimer’s Disease (AD), Huntington Disease (HD), Parkinson’s Disease (PD), and other non-neurological pathologies like obesity and Type 2 diabetes (Hojlund, Mogensen, Sahlin, & Beck-Nielsen, 2008; Lezi & Swerdlow, 2012). Upregulation of PGC1α has successfully been shown to increase OXPHOS and ameliorate several in vivo models of diseases, including age-related pathogenesis of AD, HD, and PD (Mudo et al., 2012; Tsunemi et al., 2012; Tsunemi & La Spada, 2012; Wenz, 2009).

Collectively, the ability of 5-HT$_{1F}$ receptor agonism to mitigate pathological mitochondrial damage through upregulation of PGC1α and promotion of MB should be explored as a potential therapeutic strategy to ameliorate many diseases featuring mitochondrial dysfunction in a variety of tissues.
Authorship Contributions

Participated in research design: Garrett, Whitaker, Beeson, and Schnellmann.

Conducted experiments: Garrett and Whitaker.

Performed data analysis: Garrett, Whitaker, and Schnellmann.

Wrote or contributed to the writing of the manuscript: Garrett, Whitaker, Beeson, and Schnellmann.
References:


Footnotes

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Portions of this work have been presented previously: Garrett SM, Wills LP, and Schnellmann RG (2012), “Serotonin (5-HT) 1F receptor agonism as a potential treatment for acceleration of recovery from acute kidney injury,” American Society of Nephrology Annual Meeting, Nov 1-4, 2012, San Diego, CA.
Figure Legends:

Figure 1. *Htr1f* is expressed (A) in renal proximal tubule cells (RPTC), and the 5-HT$_{1F}$ receptor protein is expressed (B) in freshly isolated renal proximal tubules (T), tubule membranes (TM), RPTC, and RPTC membranes (RM). Various concentrations of α-methyl-5-HT (αm5-HT), a non-selective 5-HT receptor agonist, and selective 5-HT$_{1F}$ receptor agonists, LY334370 and LY344864, stimulate FCCP-OCR in RPTC at 24 hr (C). Data are X + SEM, n ≥ 3. *Significantly different from vehicle (p < 0.05).

Figure 2. 5-HT$_{1F}$ receptor agonists increase mitochondrial proteins in RPTC at 24 hr. Representative immunoblots and quantification of concentration response for LY334370 (A) and LY344864 (B). ATP Synth: ATP Synthase β. Data are X + SEM, n ≥ 5. *Significantly different from vehicle (p < 0.05).

Figure 3. Knockdown of 5-HT$_{1F}$ receptor protein in RPTC by siRNA transfection at 72 hr. A. Representative immunoblot and quantification of 5-HT$_{1F}$ receptor protein knockdown. B. Mitochondrial protein levels after 5-HT$_{1F}$ receptor knockdown. Decreased 5-HT$_{1F}$ receptor levels block LY334370- and LY344864-induced MB (C). RPTC were treated with scramble or siRNA for 72 hr and treated with LY334370 or LY344864 for 24 hr. Unt, untreated; Scr, scrambled. Data are X + SEM, n ≥ 3. *Significantly different from Scr or 5-HT$_{1F}$ receptor agonist treatment (p < 0.05).
Figure 4. Gene expression of PGC1α, Cox1, NDUFB8, and mitochondrial copy number after selective 5-HT$_{1F}$ receptor agonist LY344864 (2 mg/kg) in kidney cortex at 1 hr, 8 hr, and 24 hr (A) and after selective 5-HT$_{1F}$ receptor agonist LY344864 at 1 mg/kg every 8 hr (1 mg/kg x 3), 2 mg/kg, and 10 mg/kg in kidney cortex at 24 hr (B). Data are X + SEM, n ≥ 3. *Significantly different from vehicle (p < 0.05).

Figure 5. A. *Htr1f* is expressed in murine heart (HT) and liver (LV). Gene expression of PGC1α, Cox1, NDUFB8, and mtDNA copy number after 5-HT$_{1F}$ receptor agonist LY344864 (2 mg/kg) in Heart (B) and Liver (C) at 1 hr, 8 hr, and 24 hr. Data are X + SEM, n ≥ 3. *Significantly different from vehicle (p < 0.05).

Figure 6. Blood urea nitrogen (24 hr and 144 hr, A), renal cortical KIM-1 (144 hr, B), and renal mtDNA copy number (144 hr, C) in murine I/R-AKI model. Data are X + SEM, n ≥ 4. *p<0.05 vs. sham, #p<0.05 vs. I/R + vehicle.
Figure 2

A

LY334370

ATP Synth
Cox1
NDUFB8
GAPDH

Protein Expression (Relative to Vehicle)

B

LY344864

ATP Synth
Cox1
NDUFB8
GAPDH

Protein Expression (Relative to Vehicle)
Figure 4

(A) Gene expression (relative to vehicle) for PGC1α, Cox1, and NDUFB8 mtDNA copy number.

(B) Gene expression (relative to vehicle) for PGC1α, Cox1, and NDUFB8 mtDNA copy number.

* indicates significant differences from vehicle control.
Figure 5

A

<table>
<thead>
<tr>
<th>M</th>
<th>HT</th>
<th>LV</th>
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</thead>
<tbody>
<tr>
<td>400 bp</td>
<td>362 bp</td>
<td>300 bp</td>
</tr>
</tbody>
</table>

B

- Gene Expression (Relative to Vehicle)
- 1 hr
- 8 hr
- 24 hr

C

- Gene Expression (Relative to Vehicle)
- PGC1a
- Cox1
- NDUFB8 mtDNA Copy #
Figure 6

A

![BUN (mg/dL) vs. Hours after I/R graph]

- No surgery
- Sham surgery
- I/R + Vehicle
- I/R + LY344864

B

![Western blot images for KIM-1 and β-actin]

- No surgery
- Sham surgery
- I/R + Veh
- I/R + LY

KIM-1: 65 kDa
β-actin: 42 kDa

C

![mtDNA Copy Number bar graph]

- Sham
- I/R + Veh
- I/R + LY

mtDNA Copy Number (Relative to Sham)