5-Hydroxytryptamine Receptor Stimulation of Mitochondrial Biogenesis

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ABSTRACT

Mitochondrial dysfunction is both a cause and target of reactive oxygen species during ischemia-reperfusion, drug, and toxicant injury. After injury, renal proximal tubular cells (RPTC) recover mitochondrial function by increasing the expression of the master regulator of mitochondrial biogenesis, peroxisome-proliferator-activated-receptor- γ -coactivator-1 α (PGC-1 α). The goal of this study was to determine whether 5-hydroxytryptamine (5-HT) receptor agonists increase mitochondrial biogenesis and accelerate the recovery of mitochondrial function. Reverse transcription-polymerase chain reaction analysis confirmed the presence of 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} receptor mRNA in RPTC. The 5-HT₂ receptor agonist 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane hydrochloride (DOI; 3–10 μ M)

increased PGC-1 α levels, expression of mitochondrial proteins ATP synthase β and NADH dehydrogenase (ubiquinone) 1 β subcomplex 8 (NDUFB8), MitoTracker Red staining intensity, cellular respiration, and ATP levels through a 5-HT receptor and PGC-1 α -dependent pathway. Similar effects were observed with the 5-HT₂ agonist *m*-chlorophenylpiperazine and were blocked by the 5-HT₂ antagonist 8-[3-(4-fluorophenoxy) propyl]-1-phenyl-1,3,8-triazaspiro[4,5]decan-4-one (AMI-193). In addition, DOI accelerated the recovery of mitochondrial function after oxidant-induced injury in RPTC. This is the first report to demonstrate 5-HT receptor-mediated mitochondrial biogenesis, and we suggest that 5-HT-agonists may be effective in the treatment of mitochondrial and cell injury.

Mitochondrial dysfunction is a common consequence of ischemia-reperfusion, drug, and toxicant-induced renal injury (Campbell and al-Nasser, 1996; Feldkamp et al., 2005; Honda et al., 2005). It is interesting to note that oxidant-induced mitochondrial dysfunction recovers spontaneously in primary cultures of renal proximal tubular cells (RPTC) in culture, demonstrating the presence of an endogenous mechanism to promote the recovery of mitochondrial function (Nowak et al., 1998; Rasbach and Schnellmann, 2007b). For example, after *t*-butyl-hydroperoxide (TBHP) exposure, RPTC mitochondria undergo lysosomal autophagy, effec-

tively reducing total RPTC mitochondrial volume (Rasbach and Schnellmann, 2007b). The recovery of mitochondrial function in RPTC after oxidant injury is temporally related to expression of the mitochondrial biogenesis regulator peroxisome-proliferator-activated-receptor- γ -coactivator-1 α (PGC-1 α) (Puigserver et al., 1998; Scarpulla, 2002; Rasbach and Schnellmann, 2007b). The expression of PGC-1 α after oxidant injury in RPTC is dependent upon the activation of Src, p38 mitogen-activated protein kinase, and the epidermal growth factor receptor signaling cascades that have also been implicated in postinjury migration and proliferation responses (Yano et al., 1999; Zhuang et al., 2005).

To determine whether increased mitochondrial biogenesis before oxidant injury was protective or potentiated oxidant injury, adenoviral overexpression of PGC-1 α in RPTC was used to activate the mitochondrial biogenesis program and increase mitochondrial content before oxidant injury. Depending on the oxidant, increased mitochondria either had

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ABBREVIATIONS: RPTC, renal proximal tubular cell(s); TBHP, *t*-butyl-hydroperoxide; PGC-1 α , peroxisome-proliferator-activated-receptor- γ -coactivator-1 α ; SIRT1, silent mating type information regulation 2 homolog *Saccharomyces cerevisiae*; PCR, polymerase chain reaction; 5-HT, 5-hydroxytryptamine; RT-PCR, reverse transcription-polymerase chain reaction; AMI-193, 8-[3-(4-fluorophenoxy) propyl]-1-phenyl-1,3,8-triazaspiro[4,5]decan-4-one; NDUFB8, NADH dehydrogenase (ubiquinone) 1 β subcomplex 8; DOI, 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane hydrochloride; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; RNAi, RNA interference; MG-132, *N*-benzoyloxycarbonyl (Z)-Leu-Leu-leucinal; PBS, phosphate-buffered saline.

no effect or potentiated cellular injury. In contrast, increased mitochondrial biogenesis after oxidant injury accelerated the recovery of mitochondrial functions and ATP-dependent cellular functions (Rasbach and Schnellmann, 2007a). We suggest that targeting mitochondrial biogenesis is a viable therapeutic option to promote the recovery of mitochondrial and cellular functions after injury (Rasbach and Schnellmann, 2007a,b, 2008).

Several signaling molecules have been shown to regulate the expression of PGC-1 α in response to a variety of stimuli, including nitric oxide, p38 mitogen-activated protein kinase, the tyrosine kinase Src, the epidermal growth factor receptor, cAMP response element-binding protein, activating transcription factor-2, myocyte enhancer factor-2, calcium/calmodulin-dependent protein kinases, calcineurin A, AMP-activated protein kinase, and SIRT1 (Herzig et al., 2001; Schaeffer et al., 2004; Nemoto et al., 2005; Jäger et al., 2007). Because PGC-1 α is responsive to stimuli such as cold exposure, lipopolysaccharide, oxidative stress, caloric restriction, adrenergic stimulation, exercise, and mitochondrial dysfunction, we believed that PGC-1 α expression and activity may be amenable to pharmacological manipulation (Herzig et al., 2001; Zong et al., 2002; Suliman et al., 2004; Akimoto et al., 2005; Nemoto et al., 2005; Rasbach and Schnellmann, 2007b).

Several groups have developed strategies designed to increase the expression and activity of PGC-1 α . Recent approaches have targeted the protein deacetylase SIRT1, a known PGC-1 α activator (Baur et al., 2006; Lagouge et al., 2006; Rasbach and Schnellmann, 2008). Pharmacologic activation of SIRT1 promotes the deacetylation of PGC-1 α and leads to an increase in mitochondrial number and function. For example, we reported several differentially substituted isoflavones and isoflavone derivatives promote mitochondrial biogenesis through the activation of SIRT1 and the subsequent deacetylation and activation of PGC-1 α . However, such responses only occur with either high doses or longer exposure times (48 h) (Baur et al., 2006; Lagouge et al., 2006; Rasbach and Schnellmann, 2008). Furthermore, many known SIRT1 activators are naturally derived compounds that have very low bioavailability and therefore are not favorable therapeutic candidates (Karakaya, 2004).

Spiegelman and colleagues recently developed a quantitative PCR screen to identify inducers of PGC-1 α expression (Arany et al., 2008). This screening technique demonstrated that PGC-1 α expression can be regulated by microtubule and protein synthesis inhibitors (Arany et al., 2008). Although these compounds do not harbor significant therapeutic potential, the technique demonstrates the ability to screen for inducers of PGC-1 α , and screening larger libraries may ultimately provide new useful inducers of PGC-1 α .

Because the role of 5-HT receptors in mitochondrial biogenesis has not been fully investigated and a receptor-targeted approach to increasing mitochondrial biogenesis is an attractive therapeutic strategy, we hypothesized that 5-HT receptor agonists may produce mitochondrial biogenesis in a variety of organs, including the kidney. Harris and colleagues have recently identified and characterized a putative intrarenal serotonergic system and have shown proximal tubules to contain 5-HT receptors, including type 2B, using RT-PCR (Xu et al., 2007).

Materials and Methods

Reagents. AMI-193 was purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO). Antibodies to ATP synthase- β subunit, NDUFB8, and glyceraldehyde-3-phosphate dehydrogenase were purchased from Abcam (Cambridge, MA), Invitrogen (Carlsbad, CA), and Fitzgerald Antibodies (Concord, MA), respectively. The antibody to PGC-1 α (H300) was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). We have reported previously the mitochondrial complex 1 protein ND6 as a marker of mitochondrial biogenesis (Rasbach and Schnellmann, 2007a,b, 2008). We have recently been informed by the manufacturer of the antibody (Invitrogen), that it has been confirmed that the former ND6 antibody actually recognizes the mitochondrial complex 1 protein NDUFB8. Thus, NDUFB8 is a marker of mitochondrial biogenesis. All secondary antibodies were purchased from Pierce Chemical (Rockford, IL). Anti-PGC-1 α was used at 1:300, whereas all other antibodies were used at 1:1000.

Isolation and Culture of Renal Proximal Tubules. Female New Zealand White rabbits (2 kg) were purchased from Myrtle's Rabbitry (Thompson Station, TN). RPTC were isolated using the iron oxide perfusion method and grown in 35-mm tissue culture dishes under improved conditions as described previously (Nowak and Schnellmann, 1996). The culture medium was a 1:1 mixture of Dulbecco's modified Eagle's medium/Ham's F-12 (without glucose, phenol red, or sodium pyruvate) supplemented with 15 mM HEPES buffer, 2.5 mM L-glutamine, 1 μ M pyridoxine HCl, 15 mM sodium bicarbonate, and 6 mM lactate. Hydrocortisone (50 nM), selenium (5 ng/ml), human transferrin (5 μ g/ml), bovine insulin (10 nM), and L-ascorbic acid-2-phosphate (50 μ M) were added daily to fresh culture medium. Confluent RPTC were used for all experiments. RPTC monolayers were treated with various compounds or diluent (dimethyl sulfoxide) for 24 h.

Reverse Transcription-Polymerase Chain Reaction. Total RNA extraction from RPTC was performed using TRIzol RNA extract reagent (Invitrogen). RNA (3 μ g) was subjected to first-strand cDNA synthesis using oligo(dT)₁₈ and ThermoScript Reverse transcriptase (Invitrogen) in a 20- μ l total reaction volume. Amplification of cDNA (2 μ l) was carried out using GoTaq Green Master mix (Promega, Madison, WI) with 5-HT₂ receptor subtype-specific sense and antisense primers (Table 1). Thermal cycling conditions were started with one cycle at 94°C for 30 s and then 57°C for 30 s for primer annealing, 72°C for 45 s, and final extension for 10 min at 72°C. PCR was performed using a programmed thermocycler (Eppendorf AG, Hamburg, Germany). The number of amplification cycles and their fragment size are presented in Table 1. The amplified PCR products were subjected to electrophoresis using a 1.8% agarose gel and stained with ethidium bromide.

NRK-52E Cell Experiments. Normal rat kidney (NRK-52E) epithelial cells were cultured as described previously (Covington et al., 2005) in Dulbecco's modified Eagle's medium (high glucose) supplemented with 10% fetal calf serum. For measurement of mitochondrial biogenesis, NRK52-E cells were treated with 10 μ M DOI or vehicle for 24 h, stained with MitoTracker Red (200 nM) (Invitrogen) for 20 min, washed, and imaged by fluorescence microscopy. MitoTracker staining intensity was quantified using stand-alone imaging software (Alpha Innotech, San Leandro, CA). To measure PGC-1 α transcription, NRK-52E cells were transfected with the 2-kb PGC-1 α promoter with a firefly luciferase reporter (a generous gift from Bruce Spiegelman, Harvard Medical School, Boston, MA) and the constitutive pGL4.7[hRLuc/TK] *Renilla reniformis* reporter as a control for transfection efficiency. DOI was added 48 h after infection to allow for sufficient expression of reporter vectors. Promoter activity was then measured 24 h later using a dual-luciferase reporter assay system (Promega).

Oxygen Consumption. RPTC bathed in 37°C culture medium were gently detached from culture dishes with a rubber policeman

TABLE 1

Nucleotide sequences of sense and antisense primers designed for RT-PCR amplification of rabbit nuclear genome-encoded 5-HT₂ receptors (5-HT_{2A}, -2B, -2C) and the sizes of the amplified PCR products

Primer	Sequence	Cycles	Size	GenBank Accession Number
			bp	
5- <i>htr2a</i>	5'-CCT CTC ACC ACC ATG TTT CC-3' 5'-ACA CGG GCA TGA CTA GGA AC-'	30	212	NM_017254 AF076606
5- <i>htr2b</i>	5'-TCT TCA ATA AGA CAT TTC GGG A-3' 5'-GAA TGG TTG AAC TTC GGA GC-'	30	216	NM_017250
5- <i>htr2c</i>	5'-AGA TAT TTG TGC CCC GTC TG-3' 5'-GCC TTA GTC CGC GAA TTG AA-3'	30	142	NM_012765

bp, base pairs.

and transferred to a 37°C QO₂ chamber 48 h after the initial exposure to the compounds. Basal and uncoupled (FCCP; 1 μ M) RPTC QO₂ was measured polarographically using a Clark-type electrode as described previously (Nowak and Schnellmann, 1996).

Adenoviral PGC-1 α RNAi. PGC-1 α RNAi plasmid was a generous gift from Marc Montminy (Salk Institute for Biological Studies, San Diego, CA) (Koo et al., 2004). The plasmid was digested by the endonuclease restriction enzyme PAC I (New England Biolabs, Ipswich, MA) for 5 h to linearize the Ad-Track PGC-1 RNAi plasmid. Linearized plasmid was transfected into human embryonic kidney 293 cells, and the virus was propagated for 7 days and scaled up until sufficient quantities for infection were obtained. Virus was titrated to achieve 100% green fluorescent protein-positive RPTC. Virus was present in media for 24 h. Knockdown of PGC-1 α was measured 48 h after initial exposure to PGC-1 RNAi in the presence of the proteasome inhibitor MG-132 for 4 h to stabilize PGC-1 α expression.

Cell Number. Measurement of monolayer protein content over time was used to estimate cell number. RPTC monolayers were washed with PBS, solubilized in Triton buffer (0.05% Triton X-100, 100 mM Tris-base, and 150 mM NaCl, pH 7.5), and sonicated for 60 s, and then protein concentrations were determined by the bicinchoninic acid method according to the manufacturer's instructions (Pierce Chemical).

Preparation of Cell Lysates and Immunoblot Analysis. RPTC were washed twice with PBS without Ca²⁺ and Mg²⁺ and harvested in cell lysis buffer from BioVision (Mountain View, CA). Before immunoblot analysis, all cells were disrupted by sonication for 30 s. Samples were boiled and prepared for electrophoresis. Equal amounts of cellular protein lysates were separated by SDS-polyacrylamide gel electrophoresis and electrophoretically transferred to nitrocellulose membranes. After treatment with 5% skim milk or bovine serum albumin at 4°C overnight, membranes were incubated with various antibodies for 2 h and then incubated with an appropriate horseradish peroxidase-conjugated secondary antibody for 1 h. Bound antibodies were visualized by chemiluminescence detection on an imaging system (Alpha Innotech).

Measurement of ATP. ATP was measured via luciferase-mediated bioluminescence as described previously (Lundin et al., 1986). In brief, RPTC were washed three times with ice-cold PBS and subsequently lysed on ice with Triton/glycylglycine lysis buffer. Homogenates were immediately centrifuged at 14,000g for 5 min at 4°C. Supernatants were collected and kept on ice. Each sample was combined with an equal amount of luciferase reagent (ATP Bioluminescence Assay kit CLS II; Roche Diagnostics, Indianapolis, IN) and imaged immediately using an imaging system (Alpha Innotech).

Statistical Analysis. Data are presented as means $\pm$ S.E. and were subjected to one- or two-way analysis of variance as appropriate. Multiple means were compared using Student-Newman-Keuls test, with $p < 0.05$ considered to be a statistically significant difference among means. Renal proximal tubules isolated from an individual rabbit represents a single experiment ($n = 1$). Experiments were performed with two to five plates of cells and repeated until a number of at least three was reached.

Results

DOI (Fig. 1a) is a selective agonist of 5-HT₂ (Dedeoğlu and Fisher, 1991; Cumming-Hood et al., 1993). Using RT-PCR, we confirmed that primary cultures of RPTC express the 2A, 2B, and 2C subtypes of the 5-HT receptor (Fig. 1b).

Increasing concentrations of DOI increased activity of the 2-kb PGC-1 α promoter, suggesting 5-HT receptors may mediate the transcription of this mitochondrial biogenesis regulator (Fig. 2a). To determine the effects of activating 5-HT₂ receptors on mitochondrial biogenesis, increasing concentrations of DOI were added to RPTC for 24 h, and expression of mitochondrial proteins, ATP synthase β , and NDUF8 were measured as markers of mitochondrial content (Fig. 2b). Increases in mitochondrial protein expression were seen at 3 μ M DOI and were maximal at 10 μ M DOI, suggesting that

Fig. 1. 5-HT₂ receptor subtypes in RPTC. a, DOI, a 5-HT_{2B} receptor agonist. b, total RNA was extracted and subjected to RT-PCR using gene-specific sense and antisense primers to detect the gene expression of 5-HT₂ receptor subtypes in rabbit RPTC. The figure is a representative of three independent experiments.

Fig. 2. DOI increases PGC-1 α transcription and mitochondrial protein expression. RPTC and NRK-52E cells were treated for 24 h with DOI. a, activity of the 2-kb PGC-1 α promoter with firefly luciferase reporter (in NRK-52E cells) was measured using a dual-luciferase reporter assay. b, expressions of the mitochondrial proteins ATP synthase β and NDUF8 in response to DOI were measured in RPTC via immunoblot analysis. Data represent the mean of three separate experiments. Bars with different superscripts are significantly different from each other; $p < 0.05$. Blots are representative of at least three separate experiments.

activation of 5-HT receptors with DOI induces mitochondrial biogenesis.

To further verify DOI-induced mitochondrial biogenesis, a rat epithelial cell line (NRK-52E) was used. NRK-52E cells were exposed to DOI (10 μ M) for 24 h and then stained with the mitochondria-specific dye MitoTracker Red (Fig. 3a). Fluorescence microscopy revealed an $\sim$ 2-fold increase in MitoTracker staining, again suggesting that DOI is capable of

inducing the mitochondrial biogenesis program in renal cells (Fig. 3b).

RPTC treatment with DOI promoted a concentration-dependent increase in both basal and uncoupled respiration and cellular ATP concentrations (Fig. 4). Lower concentrations of DOI (1 and 3 μ M) did not effect basal respiration; however, the higher concentration (10 μ M) increased basal respiration by $\sim$ 30% (Fig. 4a). Both 1 and 3 μ M DOI in-

Fig. 3. DOI increases mitochondrial mass/number. a, NRK-52E cells were exposed to DOI (10 μ M) for 24 h, stained with MitoTracker Red, and visualized by fluorescent microscopy. b, MitoTracker staining intensity was then quantified. Data represent the mean of three separate experiments. Bars with different superscripts are significantly different from each other; $p < 0.05$.

Fig. 4. DOI increases cellular respiration and ATP in the absence of toxicity. RPTC were treated with DOI for 24 h. Basal (a) and uncoupled (FCCP; b) respiration and ATP (c) were measured with increasing concentrations of DOI. Monolayer protein (d) was quantified as a marker of cell number and viability in the presence of increasing concentrations of DOI. Data represent the mean of three separate experiments. Bars with different subscripts are significantly different from each other; $p < 0.05$.

creased uncoupled respiration, with maximal increases at 10 μ M (Fig. 4b). Similar to the changes in basal respiration, ATP concentrations were not affected at DOI concentrations of either 1 or 3 μ M, but they were increased $\sim 45\%$ by 10 μ M DOI (Fig. 4c). These data reveal that DOI promotes the biogenesis of functional mitochondria in RPTC. Consistent with these findings, RPTC exposed to 10 μ M *m*-chlorophenylpiperazine, another 5-HT₂ receptor agonist, resulted in elevated FCCP-uncoupled respiration ($130 \pm 5\%$ control) at 24 h (data not shown).

Because the mitochondrial biogenesis program is known to be initiated in response to a number of cell stresses (Suliman et al., 2004; St-Pierre et al., 2006; Rasbach and Schnellmann, 2007b; Spiegelman, 2007), total monolayer protein content was measured to ensure that DOI did not initiate proliferation or exhibit toxicity. None of the concentrations of DOI had any effect on monolayer protein content, suggesting that DOI was not overtly toxic nor induced RPTC proliferation under these experimental conditions (Fig. 4d).

To determine whether DOI increased PGC-1 α and to verify that DOI acts through a 5-HT receptor, RPTC were pretreated with the pan-5-HT receptor antagonist AMI-193 (10 μ M), and samples were analyzed for PGC-1 α expression 12 h

Fig. 5. DOI induced up-regulation of PGC-1 α is mediated by 5-HT receptors. RPTC were pretreated (30 min) with either the pan-5-HT receptor antagonist AMI-193 (10 μ M) or vehicle control and then exposed to DOI (10 μ M) for 12 h. PGC-1 α expression was then measured by immunoblot. Blots are representative of at least three separate experiments.

post-DOI (10 μ M) exposure (Fig. 5). PGC-1 α expression was up-regulated in DOI-treated RPTC; however, the DOI-mediated increase in PGC-1 α expression was attenuated by AMI-193, suggesting that DOI induced increases in PGC-1 α expression are mediated by 5-HT receptors.

To validate that DOI-induced mitochondrial biogenesis was dependent on PGC-1 α , PGC-1 α expression was first decreased $\sim 45\%$ 48 h after infection with an adenoviral PGC-1 α -RNAi compared with a scramble RNAi control (Fig. 6a). RPTC were then exposed to DOI (10 μ M) for 24 h in the presence or absence of PGC-1 α -RNAi. Exposure to DOI in-

Fig. 6. DOI induced increases in mitochondrial protein expression are mediated by PGC-1α. **a**, RPTC were exposed to adenoviral RNAi directed against PGC-1α for 24 h. PGC-1α expression was measured in the presence of MG-132 (4 h before sample collection) by immunoblot 48 h after the initial infection. **b**, RPTC in which PGC-1α expression had been reduced were exposed to DOI (10 μM) for 24 h, and the expression of mitochondrial proteins (ATP synthase β and NDUF8) was measured by immunoblot. Blots are representative of at least three separate experiments.

creased the expression of the mitochondrial proteins ATP synthase β and NDUF8, and this increase in mitochondrial proteins was blocked in RPTC in which PGC-1α had been decreased using RNAi (Fig. 6b). We suggest that DOI-mediated mitochondrial biogenesis is dependent on PGC-1α.

Because increasing PGC-1α expression and mitochondrial biogenesis subsequent to oxidant injury promotes the recovery of mitochondrial and cellular functions (Rasbach and Schnellmann, 2007a), we sought to examine the effects of

DOI on RPTC mitochondrial function after exposure to the model oxidant TBHP (200 μM for 30 min). DOI (10 μM) was added to RPTC 6 h after TBHP exposure to allow for the injury process to take place, and respiration was measured 24 h later. TBHP induced mitochondrial dysfunction as reflected by decreases in both basal and uncoupled respiration (Fig. 7). Treatment of RPTC with DOI promoted the return of both basal and uncoupled respiration to control values within 24 h after the initial exposure to TBHP, whereas untreated

Fig. 7. DOI accelerates recovery of mitochondrial function after TBHP exposure. RPTC were treated with DOI (10 μM) 6 h after TBHP injury (200 μM for 30 min). Mitochondrial function was assessed by monitoring basal and uncoupled (FCCP, 1 μM) respiration 24 h after DOI treatment. Different subscripts are significantly different from each other; $p < 0.05$.

RPTC still exhibited significant mitochondrial dysfunction (Fig. 7). In contrast, when RPTC were pretreated with DOI (10 μ M) for 24 h before TBHP exposure, basal and uncoupled respiration remained decreased 24 h after TBHP exposure, suggesting DOI pretreatment offers no protection from oxidant injury (Fig. 8). These data reveal that DOI-induced increases in mitochondrial biogenesis accelerate the recovery of mitochondrial function after oxidant injury, but they do not confer protection from oxidant-mediated mitochondrial dysfunction.

Discussion

Although mitochondrial dysfunction is a common consequence of ischemic- and drug-induced injuries, and other disease processes, very few therapies have been directed toward preventing or promoting the recovery of mitochondrial injury. Furthermore, mitochondrial number is decreased after oxidative stress observed in models of ischemia-reperfusion injury or from the cumulative oxidative stress associated with aging (Lemasters, 2005; Rasbach and Schnellmann, 2007b). Several disease models or toxicants decrease the expression of PGC-1 α and are associated with impaired mitochondrial biogenesis (Portilla et al., 2002; Priault et al., 2005). Therefore, disease states or injury models associated with mitochondrial dysfunction and/or impaired biogenesis of mitochondria is therefore attractive targets for therapeutics aimed at stimulating the mitochondrial biogenesis pathway.

In this study, we describe a novel 5-HT-dependent mechanism controlling the biogenesis of mitochondria. The 5-HT₂ receptor agonist DOI increased PGC-1 α promoter activity, suggesting that agonism of 5-HT receptors regulates the activity of PGC-1 α at the transcriptional level. The increase in PGC-1 α promoter activity was observed with 10 μ M DOI, and this concentration of DOI maximally increased mitochondrial biogenesis as demonstrated by the concentration-dependent increase in mitochondrial protein expression (ATP synthase β and NDUFB8), MitoTracker Red staining inten-

sity, cellular respiration, and ATP concentrations. At 3 μ M, DOI increased some markers of mitochondrial biogenesis. The 5-HT₂ receptor agonist *m*-chlorophenylpiperazine also increased mitochondrial biogenesis. The pan-5-HT receptor antagonist (AMI-193) blocked DOI-mediated increases in PGC-1 α , demonstrating that DOI acted through 5-HT receptors. Although DOI is selective for 5-HT₂ receptors, the EC₅₀ value for DOI in isolated receptor systems is in the nanomolar range. This observation provides evidence that DOI may act as a more promiscuous activator of 5-HT receptor subtypes in this system and that the observed increases in mitochondrial biogenesis may be through other 5-HT receptors.

Nebigil et al. focused on the role of 5-HT_{2B} receptor signaling in the heart (Nebigil et al., 2003a,b; Nebigil and Maroteaux, 2003). Overexpression of 5-HT_{2B} receptors in the heart lead to increases in mitochondrial enzyme activities in isolated ventricular slices (Nebigil et al., 2003b). In addition, these mice developed severe cardiac hypertrophy and a dilated cardiomyopathy as a result of mitochondrial proliferation and increased cell number and size, which were similar to the phenotype observed in transgenic mice with cardiac specific overexpression of PGC-1 α (Lehman et al., 2000; Lehman and Kelly, 2002). Specific knockout of 5-HT_{2B} receptors in the heart resulted in marked decreases in succinate dehydrogenase and cytochrome oxidase activities in ventricular sections and reductions in mitochondrial number associated with increased myocardial cell death (Nebigil et al., 2003a).

Because PGC-1 α is known to promote mitochondrial biogenesis in RPTC (Rasbach and Schnellmann, 2007a,b, 2008), we validated that the observed increase in PGC-1 α expression was responsible for mediating DOI-induced mitochondrial biogenesis. PGC-1 α expression was reduced $\sim$ 45% using an adenoviral PGC-1 α -RNAi for 48 h. The knockdown of PGC-1 α was sufficient to prevent DOI-induced increases in the expression of both nuclear- (ATP synthase β) and mitochondrial (NDUFB8)-encoded mitochondrial proteins, revealing that DOI increases mitochondrial biogenesis through a PGC-1 α -dependent mechanism.

Cell stress is often associated with compensatory responses

Fig. 8. DOI pretreatment does not protect against oxidant-induced mitochondrial dysfunction. RPTC were treated with DOI (10 μ M) for 24 h, at which time basal and uncoupled respiration was measured (gray bars). RPTC were then exposed to TBHP (200 μ M; 30 min), and basal and uncoupled respiration were measured 24 h after TBHP exposure in DOI or vehicle-pretreated cells (black bars).

such as mitochondrial biogenesis (Rasbach and Schnellmann, 2007b; Spiegelman, 2007). We determined whether DOI was toxic to RPTC at any concentrations shown to promote mitochondrial biogenesis. Neither morphological changes (data not shown) nor a change in cell number was observed, suggesting DOI did not induce injury in RPTC.

Increasing the expression of PGC-1 α after oxidant injury is known to accelerate the recovery of RPTC mitochondrial and cellular functions (Rasbach and Schnellmann, 2007a). Thus, we determined the effect of DOI-mediated increases in PGC-1 α expression and mitochondrial biogenesis on the recovery of mitochondrial function after injury. The addition of DOI 6 h post-TBHP exposure increased the rate of recovery of both basal and uncoupled cellular respiration 24 h after injury. Pretreatment of RPTC with DOI for 24 h, however, did not protect against TBHP-induced mitochondrial dysfunction, which is consistent with our previous study that showed overexpressing PGC-1 α before oxidant injury did not protect mitochondrial function (Rasbach and Schnellmann, 2007a). Therefore, we suggest that DOI or other 5-HT receptor agonists may be an effective therapeutic option to accelerate the recovery of renal function subsequent to acute kidney injury.

Identifying a receptor-mediated approach to increase the expression of PGC-1 α and promote mitochondrial biogenesis may be a viable therapeutic option for the treatment of a variety of disorders associated with mitochondrial dysfunction. Although chronic overexpression of 5-HT2B or PGC-1 α in the heart has pathological effects (Lehman et al., 2000; Lehman and Kelly, 2002), limited exposure to pharmacological activators of mitochondrial biogenesis during periods of mitochondrial dysfunction may result in improved tissue function with limited untoward effects. Furthermore, agents that increase 5-HT receptor activation indirectly, such as 5-HT reuptake inhibitors, may act through mitochondrial biogenesis. Finally, targeting specific 5-HT receptors to induce mitochondrial biogenesis may provide some order of specificity for targeting the biogenesis response, as the actions would be limited to those tissues expressing specific 5-HT receptors.

References

- Akimoto T, Pohnert SC, Li P, Zhang M, Gumbs C, Rosenberg PB, Williams RS, and Yan Z (2005) Exercise stimulates Pgc-1 α transcription in skeletal muscle through activation of the p38 MAPK pathway. *J Biol Chem* **280**:19587–19593.
- Arany Z, Wagner BK, Ma Y, Chinsomboon J, Laznik D, and Spiegelman BM (2008) Gene expression-based screening identifies microtubule inhibitors as inducers of PGC-1 α and oxidative phosphorylation. *Proc Natl Acad Sci U S A* **105**:4721–4726.
- Baur JA, Pearson KJ, Price NL, Jamieson HA, Lerin C, Kalra A, Prabhu VV, Allard JS, Lopez-Lluch G, Lewis K, et al. (2006) Resveratrol improves health and survival of mice on a high-calorie diet. *Nature* **444**:337–342.
- Campbell PI and al-Nasser IA (1996) Renal insufficiency induced by cisplatin in rats is ameliorated by cyclosporin A. *Toxicology* **114**:11–17.
- Covington MD, Bayless KJ, Burghardt RC, Davis GE, and Parrish AR (2005) Ischemia-induced cleavage of cadherins in NRK cells: evidence for a role of metalloproteinases. *Am J Physiol Renal Physiol* **289**:F280–F288.
- Cumming-Hood PA, Strahlendorf JC (1993) Effects of serotonin and the 5-HT₂/1C receptor agonist DOI on neurons of the cerebellar dentate/interpositus nuclei: possible involvement of a GABAergic interneuron. *Eur J Pharmacol* **236**:457–465.
- Dedeoglu A and Fisher LA (1991) Central and peripheral injections of the 5-HT₂ agonist, 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane, modify cardiovascular function through different mechanisms. *J Pharmacol Exp Ther* **259**:1027–1034.
- Feldkamp T, Kribben A, and Weinberg JM (2005) Assessment of mitochondrial membrane potential in proximal tubules after hypoxia-reoxygenation. *Am J Physiol Renal Physiol* **288**:F1092–F1102.
- Herzig S, Long F, Jhala US, Hedrick S, Quinn R, Bauer A, Rudolph D, Schutz G, Yoon C, Puigserver P, et al. (2001) CREB regulates hepatic gluconeogenesis through the coactivator PGC-1. *Nature* **413**:179–183.
- Honda HM, Korge P, and Weiss JN (2005) Mitochondria and ischemia/reperfusion injury. *Ann N Y Acad Sci* **1047**:248–258.
- Jäger S, Handschin C, St-Pierre J, and Spiegelman BM (2007) AMP-activated protein kinase (AMPK) action in skeletal muscle via direct phosphorylation of PGC-1 α . *Proc Natl Acad Sci U S A* **104**:12017–12022.
- Karakaya S (2004) Bioavailability of phenolic compounds. *Crit Rev Food Sci Nutr* **44**:453–464.
- Koo SH, Satoh H, Herzog S, Lee CH, Hedrick S, Kulkarni R, Evans RM, Olefsky J, and Montminy M (2004) PGC-1 promotes insulin resistance in liver through PPAR- α -dependent induction of TRB-3. *Nat Med* **10**:530–534.
- Lagouge M, Argmann C, Gerhart-Hines Z, Meziane H, Lerin C, Daussin F, Messadeq N, Milne J, Lambert P, Elliott P, et al. (2006) Resveratrol improves mitochondrial function and protects against metabolic disease by activating SIRT1 and PGC-1 α . *Cell* **127**:1109–1122.
- Lehman JJ and Kelly DP (2002) Gene regulatory mechanisms governing energy metabolism during cardiac hypertrophic growth. *Heart Fail Rev* **7**:175–185.
- Lehman JJ, Barger PM, Kovacs A, Saffitz JE, Medeiros DM, and Kelly DP (2000) Peroxisome proliferator-activated receptor gamma coactivator-1 promotes cardiac mitochondrial biogenesis. *J Clin Invest* **106**:847–856.
- Lemasters JJ (2005) Selective mitochondrial autophagy, or mitophagy, as a targeted defense against oxidative stress, mitochondrial dysfunction, and aging. *Rejuvenation Res* **8**:3–5.
- Lundin A, Hasenson M, Persson J, and Pousette A (1986) Estimation of biomass in growing cell lines by adenosine triphosphate assay. *Methods Enzymol* **133**:27–42.
- Nebigil CG, Etienne N, Messaddeq N, and Maroteaux L (2003a) Serotonin is a novel survival factor of cardiomyocytes: mitochondria as a target of 5-HT_{2B} receptor signaling. *FASEB J* **17**:1373–1375.
- Nebigil CG, Jaffré F, Messaddeq N, Hickel P, Monassier L, Launay JM, and Maroteaux L (2003b) Overexpression of the serotonin 5-HT_{2B} receptor in heart leads to abnormal mitochondrial function and cardiac hypertrophy. *Circulation* **107**:3223–3229.
- Nebigil CG and Maroteaux L (2003) Functional consequence of serotonin/5-HT_{2B} receptor signaling in heart: role of mitochondria in transition between hypertrophy and heart failure? *Circulation* **108**:902–908.
- Nemoto S, Fergusson MM, and Finkel T (2005) SIRT1 functionally interacts with the metabolic regulator and transcriptional coactivator PGC-1 α . *J Biol Chem* **280**:16456–16460.
- Nowak G, Aleo MD, Morgan JA, and Schnellmann RG (1998) Recovery of cellular functions following oxidant injury. *Am J Physiol* **274**:F509–F515.
- Nowak G and Schnellmann RG (1996) L-ascorbic acid regulates growth and metabolism of renal cells: improvements in cell culture. *Am J Physiol* **271**:C2072–C2080.
- Portilla D, Dai G, McClure T, Bates L, Kurten R, Megyesi J, Price P, and Li S (2002) Alterations of PPAR α and its coactivator PGC-1 in cisplatin-induced acute renal failure. *Kidney Int* **62**:1208–1218.
- Priault M, Salin B, Schaeffer J, Vallette FM, di Rago JP, and Martinou JC (2005) Impairing the bioenergetic status and the biogenesis of mitochondria triggers mitophagy in yeast. *Cell Death Differ* **12**:1613–1621.
- Puigserver P, Wu Z, Park CW, Graves R, Wright M, and Spiegelman BM (1998) A cold-inducible coactivator of nuclear receptors linked to adaptive thermogenesis. *Cell* **92**:829–839.
- Rasbach KA and Schnellmann RG (2007a) PGC-1 α over-expression promotes recovery from mitochondrial dysfunction and cell injury. *Biochem Biophys Res Commun* **355**:734–739.
- Rasbach KA and Schnellmann RG (2007b) Signaling of mitochondrial biogenesis following oxidant injury. *J Biol Chem* **282**:2355–2362.
- Rasbach KA and Schnellmann RG (2008) Isoflavones promote mitochondrial biogenesis. *J Pharmacol Exp Ther* **325**:536–543.
- Scarpulla RC (2002) Nuclear activators and coactivators in mammalian mitochondrial biogenesis. *Biochim Biophys Acta* **1576**:1–14.
- Schaeffer PJ, Wende AR, Magee CJ, Neilson JR, Leone TC, Chen F, and Kelly DP (2004) Calcineurin and calcium/calmodulin-dependent protein kinase activate distinct metabolic gene regulatory programs in cardiac muscle. *J Biol Chem* **279**:39593–39603.
- Spiegelman BM (2007) Transcriptional control of energy homeostasis through the PGC1 coactivators. *Novartis Found Symp* **286**:3–6; discussion 6–12, 162–163, 196–203.
- St-Pierre J, Drori S, Uldry M, Silvaggi JM, Rhee J, Jäger S, Handschin C, Zheng K, Lin J, Yang W, et al. (2006) Suppression of reactive oxygen species and neurodegeneration by the PGC-1 transcriptional coactivators. *Cell* **127**:397–408.
- Suliman HB, Welty-Wolf KE, Carraway M, Tatro L, and Piantadosi CA (2004a) Lipopolysaccharide induces oxidative cardiac mitochondrial damage and biogenesis. *Cardiovasc Res* **64**:279–288.
- Xu J, Yao B, Fan X, Langworthy MM, Zhang MZ, and Harris RC (2007) Characterization of a putative intrarenal serotonergic system. *Am J Physiol Renal Physiol* **293**:F1468–F1475.
- Yano T, Yazima S, Hagiwara K, Ozasa H, Ishizuka S, and Horikawa S (1999) Activation of epidermal growth factor receptor in the early phase after renal ischemia-reperfusion in rat. *Nephron* **81**:230–233.
- Zhuang S, Yan Y, Han J, and Schnellmann RG (2005) p38 kinase-mediated transactivation of the epidermal growth factor receptor is required for differentiation of renal epithelial cells after oxidant injury. *J Biol Chem* **280**:21036–21042.
- Zong H, Ren JM, Young LH, Pypaert M, Mu J, Birnbaum MJ, and Shulman GI (2002) AMP kinase is required for mitochondrial biogenesis in skeletal muscle in response to chronic energy deprivation. *Proc Natl Acad Sci U S A* **99**:15983–15987.

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