

Proximal Tubule β_2 -Adrenergic Receptor Mediates Formoterol-Induced Recovery of Mitochondrial and Renal Function after Ischemia-Reperfusion Injury^{SI}

Robert B. Cameron, Whitney S. Gibbs, Siennah R. Miller, Tess V. Dupre, Judit Megyesi, Craig C. Beeson, and Rick G. Schnellmann

Department of Drug Discovery and Biomedical Sciences, Medical University of South Carolina, Charleston, South Carolina (R.B.C., W.S.G., C.C.B.); College of Pharmacy, University of Arizona (R.B.C., W.S.G., S.R.M., T.V.D., R.G.S.), and Southern Arizona VA Healthcare System (R.G.S.), Tucson, Arizona; and Department of Medicine, University of Arkansas for Medical Sciences, Little Rock, Arkansas (J.M.)

Received August 10, 2018; accepted January 4, 2019

ABSTRACT

Acute kidney injury (AKI) is the rapid loss of renal function after an insult, and renal proximal tubule cells (RPTCs) are central to the pathogenesis of AKI. The β_2 -adrenergic receptor (β_2 AR) agonist formoterol accelerates the recovery of renal function in mice after ischemia-reperfusion injury (IRI) with associated rescue of mitochondrial proteins; however, the cell type responsible for this recovery remains unknown. The role of RPTCs in formoterol-induced recovery of renal function was assessed in a proximal tubule-specific knockout of the β_2 AR (γ GT-Cre:ADRB2^{Flox/Flox}). These mice and wild-type controls (ADRB2^{Flox/Flox}) were subjected to renal IRI, followed by once-daily dosing of formoterol beginning 24 hours post-IRI and euthanized at 144 hours. Compared with ADRB2^{Flox/Flox} mice, γ GT-Cre:ADRB2^{Flox/Flox} mice had decreased renal cortical mRNA expression of the β_2 AR. After IRI, formoterol treatment

restored renal function in ADRB2^{Flox/Flox} but not γ GT-Cre:ADRB2^{Flox/Flox} mice as measured by serum creatinine, histopathology, and expression of kidney injury marker-1 (KIM-1). Formoterol-treated ADRB2^{Flox/Flox} mice exhibited recovery of mitochondrial proteins and DNA copy number, whereas γ GT-Cre:ADRB2^{Flox/Flox} mice treated with formoterol did not. Analysis of mitochondrial morphology by transmission electron microscopy demonstrated that formoterol increased mitochondrial number and density in ADRB2^{Flox/Flox} mice but not in γ GT-Cre:ADRB2^{Flox/Flox} mice. These data demonstrate that proximal tubule β_2 AR regulates renal mitochondrial homeostasis. Formoterol accelerates the recovery of renal function after AKI by activating proximal tubule β_2 AR to induce mitochondrial biogenesis and demonstrates the overall requirement of RPTCs in renal recovery.

Introduction

Acute kidney injury (AKI) is a rapid loss of renal function that occurs in more than 20% of hospitalized patients and has a mortality rate of 25% (Susantitaphong et al., 2013; Hoste et al., 2015). AKI has numerous causes, including hypotension, nephrotoxic drug administration, and renal ischemia-reperfusion injury (IRI) (Moore et al., 2018). Unfortunately, treatment of AKI remains limited to supportive care and renal replacement therapy.

The difficulty in treating AKI is the numerous cell types involved, including immune cells (Jang and Rabb, 2015),

endothelial cells (Molitoris, 2014), and the renal epithelium (Liu et al., 2018). Renal proximal tubule cells (RPTCs) are a highly oxidative and regenerative cell type that plays a central role in the pathogenesis of AKI (Sekine et al., 2012; Ralton and Parikh, 2016). RPTCs exhibit mitochondrial fragmentation and dysfunction with persistent suppression of mitochondrial biogenesis (MB) after AKI (Brooks et al., 2009; Tran et al., 2011; Funk and Schnellmann, 2012). Transgenic mouse models have shown that decreased MB worsens AKI, whereas increased MB accelerates recovery (Tran et al., 2016).

Drugs that increase MB accelerate recovery from AKI with concomitant rescue of mitochondrial protein expression and function (Wills et al., 2012). One such drug is the Food and Drug Administration–approved β_2 -adrenergic receptor (β_2 AR) agonist formoterol (Wills et al., 2012). We reported that formoterol treatment restored renal function with concomitant increases in mitochondrial protein expression and function after AKI in mice (Jesinkey et al., 2014). We also elucidated the mechanism of formoterol-induced MB in RPTCs (Cameron et al., 2017).

Portions of this work were presented at Experimental Biology 2018. Primary Laboratory: Schnellmann Laboratory, University of Arizona. R.B.C. is funded by [F30 DK104550] and [T32 GM008716] (National Institutes of Health). W.S.G. is funded by [T32 DK083262]. T.V.D. is funded by [T32 HL007249]. C.C.B. is funded by [P20 GM103542] (NIH). R.G.S. is funded by [R01 GM084147] and [1BX000851] (Department of Veterans Affairs). <https://doi.org/10.1124/jpet.118.252833>.
^{SI} This article has supplemental material available at jpet.aspetjournals.org.

ABBREVIATIONS: AKI, acute kidney injury; β_2 AR, β_2 -adrenergic receptor; DMSO, dimethylsulfoxide; Drp1, dynamin-related protein 1; IRI, ischemia-reperfusion injury; KIM-1, kidney injury marker-1; KO, knockout; MB, mitochondrial biogenesis; Mfn2, mitofusin 2; NDUFS1, NADH:ubiquinone oxidoreductase core subunit S1; PCR, polymerase chain reaction; PGC-1 α , peroxisome proliferator-activated receptor gamma coactivator-1 α ; RPTC, renal proximal tubule cell; Scr, serum creatinine; WT, wild-type.

Formoterol binding to the β_2 AR results in the release of G $\beta\gamma$ heterodimer, the activation of protein kinase B, the phosphorylation of endothelial nitric oxide synthase, and increased soluble guanylyl cyclase activity and cGMP. This pathway increased peroxisome proliferator-activated receptor gamma coactivator-1 α (PGC-1 α), the master regulator of MB (Puigserver et al., 1998), with concomitant induction of MB.

Because the β_2 AR is ubiquitously expressed (e.g., T cells, macrophages, neutrophils, endothelial cells, and RPTCs), it is not clear which cell(s) are responsible for formoterol-induced MB and recovery of renal function after IR-induced AKI. The goal of this study was to determine the specific role of RPTC β_2 AR in AKI and formoterol-induced recovery of mitochondrial and renal function using a mouse with proximal tubule-specific deletion of the β_2 AR (Iwano et al., 2002).

Materials and Methods

Animal Use. ADRB2^{Flox/Flox} mice (Hinoi et al., 2008) were a generous gift from Drs. Zhi Zhong and Gerard Karsenty and were mated with γ GT-Cre mice (Iwano et al., 2002), a generous gift from Dr. Leslie Gewin, to generate γ GT-Cre:ADRB2^{Flox/Flox} mice. These mice were generated on a C57Bl/6 background, and male 8- to 10-week-old littermates were subjected to bilateral renal ischemia-reperfusion injury as previously described (Funk and Schnellmann, 2012). Dosing was initiated 24 hours after reperfusion, and mice were given a daily injection of 0.3 mg/kg formoterol fumarate dihydrate (F9552; Sigma-Aldrich, St. Louis, MO) or vehicle [0.3% dimethylsulfoxide (DMSO) in normal saline] via intraperitoneal injection. Blood was collected by retro-orbital bleeding puncture, and serum creatinine (SCr) level was determined using the Creatinine Enzymatic Reagent Assay kit (Diazyme Laboratories, Inc., La Jolla, CA) according to the manufacturer's protocol. All experiments were carried out in accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health. All procedures were approved by the University of Arizona Institutional Animal Care and Use Committee, and appropriate efforts were made to reduce animal suffering.

Nucleic Acid Isolation and Quantitative Polymerase Chain Reaction. RNA was extracted from frozen renal cortex in TRIzol isolated using a phenol-based centrifugation method (Life Technologies, Grand Island, NY). cDNA was reverse-transcribed using the iScript Advanced cDNA Synthesis Kit (BioRad, Hercules, CA) and added to a real-time SYBR Green quantitative polymerase chain reaction master mix (BioRad). Changes in gene expression were calculated based on the $\Delta\Delta$ threshold cycle method. Primers are reported in Supplemental Materials.

Mouse-tail tips were lysed using Direct PCR (polymerase chain reaction) lysis reagent (ViaGen, Inc., Cedar Park, TX). Genomic DNA was amplified using Promega 2X PCR Master Mix (Promega, Madison, WI) in accordance with manufacturer's protocols. Amplified DNA was separated on a 2.5% agarose gel and visualized by ethidium bromide fluorescence. ADRB2^{Flox} alleles were distinguished from ADRB2⁺ alleles based on fragment size as previously described (Hinoi et al., 2008).

To measure mtDNA copy number, DNA was extracted from frozen renal cortex using the DNeasy Blood and Tissue kit (QIAGEN, Valencia, CA). PCR products were amplified from 5 ng of cellular DNA using a real-time SYBR Green quantitative PCR (BioRad). For estimation of mtDNA, the NADH dehydrogenase subunit 6 (ND6) gene was used and normalized to β -actin.

Protein Isolation and Immunoblotting. Frozen renal cortex was suspended in protein lysis buffer (1% Triton X-100, 150 mM NaCl, and 10 mM Tris-HCl, pH 7.4; 1 mM EDTA; 1 mM EGTA; 2 mM sodium orthovanadate; 0.2 mM phenylmethylsulfonyl fluoride; 1 mM HEPES, pH 7.6) containing protease inhibitors and phosphatase inhibitors (Sigma-Aldrich). After sonication, protein was quantified using a bicinchoninic acid assay, subjected to SDS-PAGE, transferred onto

nitrocellulose membranes, and incubated with primary and secondary antibodies. Membranes were detected using chemiluminescence and processed using ImageJ (National Institutes of Health, Bethesda, MD) software. Antibodies are reported in Supplemental Materials.

Electron Microscopy. Renal cortex was fixed and sectioned for transmission electron microscopy. Images were viewed by FEI Tecnai Spirit microscope operated at 100 kV and captured using an AMT 4 Mpixel camera. Mitochondrial count and morphology were analyzed using the Trainable Weka Segmentation plugin in ImageJ.

Histopathology. Kidney sections (approximately 5 to 6 μ m) from animals 144 hours after IR or sham surgery were stained with H&E and periodic acid-Schiff, and the degree of morphologic changes was determined by light microscopy in a blinded fashion. Loss of brush border and necrosis were chosen as indicators of morphologic damage to the kidney. These measures were evaluated on a scale from 0 to 4, which ranged from not present (0), mild (1), moderate (2), severe (3), and very severe (4).

Statistical Analysis. Data are expressed as means \pm S.E.M. ($n \geq 3$) for all experiments. Each n represents a different animal. Multiple comparisons of normally distributed data were analyzed by two-way analysis of variance. Single comparisons were analyzed using a t test where appropriate. The criterion for statistical differences was $P < 0.05$ for all comparisons.

Results

RPTC-specific deletion of the β_2 AR in mice was achieved by breeding γ GT-Cre mice with ADRB2^{Flox/Flox} (wild-type, WT) mice to create a γ GT-Cre:ADRB2^{Flox/Flox} mouse (knockout, KO) (Fig. 1A). The presence of ADRB2^{Flox} alleles was determined by DNA electrophoresis. Tail tips were digested, and genomic DNA was amplified. As previously described (Hinoi et al., 2008), the ADRB2^{Flox} allele has a longer sequence than the ADRB2⁺ allele, allowing for discrimination of ADRB2^{+/+} (one low band), ADRB2^{Flox/+} (one high band and one low band), and ADRB2^{Flox/Flox} (one high band) mice. Deletion of ADRB2 and appropriate expression of the Cre transgene was assessed by qPCR using renal cortical DNA. Expression of ADRB2 was assessed by RT-qPCR using renal cortical mRNA. Consistent with the loss of β_2 AR in RPTCs, KO mice had an 80% reduction in renal cortical ADRB2 DNA and mRNA expression and increased γ GT-Cre DNA expression relative to WT mice (Fig. 1, B–D).

The role of RPTC β_2 AR on recovery from AKI was determined in KO and WT mice subjected to renal IRI, followed by treatment with vehicle or formoterol (0.3 mg/kg, i.p) after 24 hours and then daily for 144 hours. WT and KO mice had similar increases in SCr at 24 hours, indicating no difference in initial injury (Fig. 2A). As previously described, (Jesinkey et al., 2014) WT mice treated with formoterol exhibited recovery from AKI at 144 hours as measured by decreases in SCr and renal cortical kidney injury marker-1 (KIM-1) protein (Fig. 2, B and F). In contrast, KO mice treated with formoterol did not exhibit decreases in SCr and renal cortical KIM-1 at 144 hours. These findings were confirmed by histopathology in that formoterol-treated WT mice had less necrosis than did vehicle-treated animals, whereas formoterol failed to decrease necrosis in KO mice at 144 hours (Fig. 2, C and D). Together, these findings provide evidence that, after AKI, formoterol exerts its effects on renal recovery by activating the RPTC β_2 AR.

RPTCs have high mitochondrial content to maintain proper solute transport across the tubular lumen (Nakamura et al., 2014; Chevalier, 2016; Ralto and Parikh, 2016). After AKI, MB

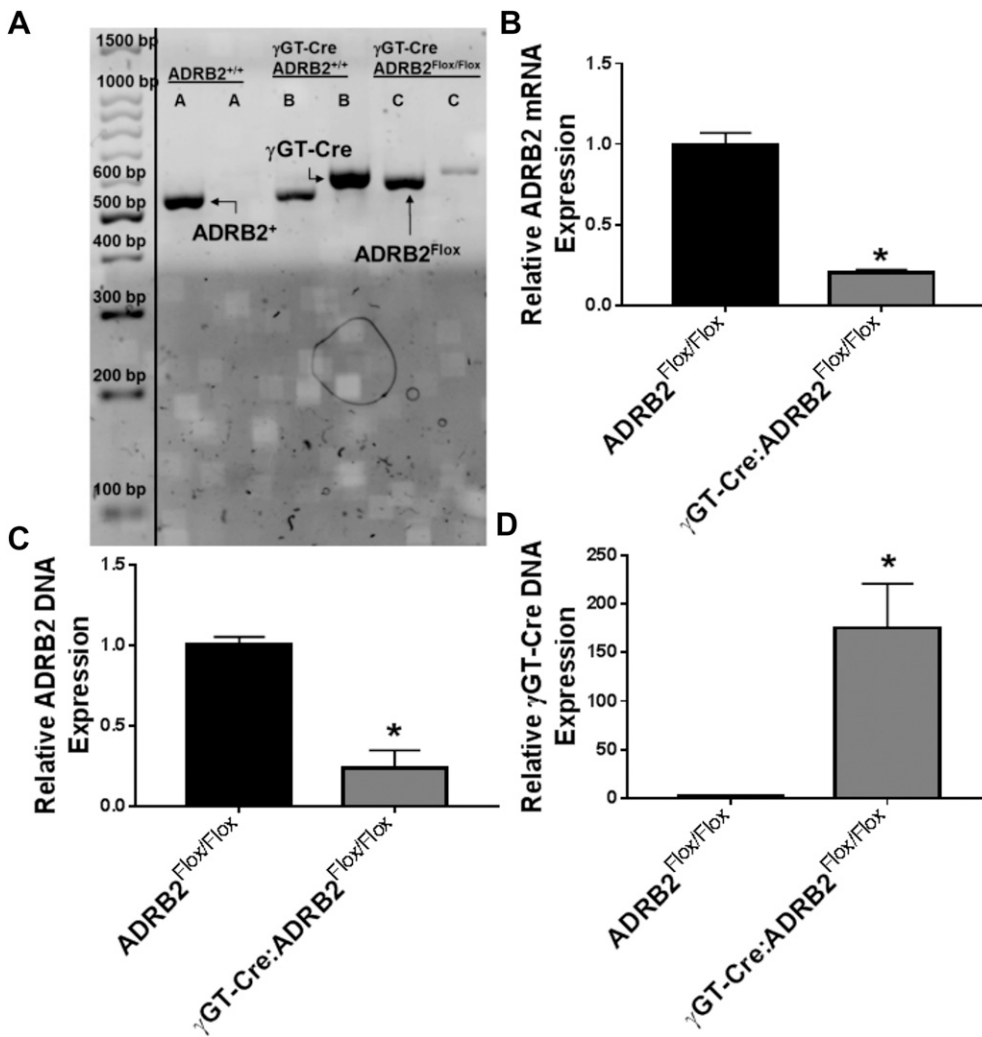


Fig. 1. γ GT-Cre:ADRB2^{Flox/Flox} mice have proximal tubule specific deletion of the β_2 adrenergic receptor. (A) DNA electrophoresis of: an ADRB2^{+/+} mouse homozygous for ADRB2⁺ (lane 1) and not expressing γ GT-Cre (lane 2); a mouse γ GT-Cre:ADRB2^{+/+} homozygous for ADRB2⁺ (lane 3) and expressing γ GT-Cre (lane 4); and a γ GT-Cre:ADRB2^{Flox/Flox} mouse homozygous for ADRB2^{Flox} (lane 5) and expressing γ GT-Cre (lane 6). (B) RT-PCR of ADRB2 mRNA in ADRB2^{Flox/Flox} and γ GT-Cre:ADRB2^{Flox/Flox} mice. (C and D) PCR of ADRB2 (C) and γ GT-Cre (D) DNA. All samples are from the renal cortex. Mean \pm S.E.M. $n = 4$ to 5 , * $P < 0.05$, Student's t test.

is persistently suppressed, and recovery of mitochondrial content is associated with recovery of renal function and improved outcomes (Tran et al., 2011). The effects of RPTC β_2 AR on mitochondrial content were assessed by measuring mtDNA and mitochondrial proteins using quantitative PCR and immunoblot analysis, respectively. Formoterol restored mtDNA copy number in WT but not in KO mice after IRI (Fig. 3A). Similarly, KO mice subjected to IRI and treated with formoterol demonstrated no recovery of nuclear-encoded NADH:ubiquinone oxidoreductase core subunit S1 (NDUFS1) and the mitochondrial-encoded cytochrome c oxidase subunit 1 (COX1), electron transport chain proteins, and markers of MB (Fig. 3B). Thus, activation of RPTC β_2 AR by formoterol rescues markers of MB after AKI. Interestingly, KO shams had elevated expression of NDUFS1, which suggests that the β_2 AR may regulate mitochondrial homeostasis in healthy RPTCs.

In addition to the restoration of electron transport chain proteins, formoterol restored mitochondrial fission and fusion proteins dynamin-related protein 1 (Drp1) and mitofusin (Mfn2), respectively, in WT but not in KO mice (Fig. 3C). Because Drp1 and Mfn2 affect mitochondrial dynamics, we assessed mitochondrial morphology using transmission electron microscopy. Electron micrographs were obtained, and

mitochondrial morphology and number were quantified using ImageJ and the Trainable Weka Segmentation plugin. We observed no changes in mitochondrial form factor or individual mitochondrial area, suggesting that formoterol did not affect mitochondrial fission and fusion at 144 hours (Fig. 4, A–C); however, in WT mice, renal cortical mitochondrial number and total mitochondria area decreased after IRI, and formoterol restored these parameters, indicating that formoterol induced MB (Fig. 4, D and E). Sham-operated KO mice had fewer mitochondria, providing evidence that RPTC β_2 AR regulates mitochondrial homeostasis under physiologic conditions. Together, these data indicate that formoterol activates RPTC β_2 AR to induce MB and accelerate recovery of renal function after AKI.

Discussion

We demonstrated that RPTC β_2 AR is responsible for the effects of formoterol on renal function after AKI. Previous studies in septic AKI demonstrated that overexpression or activation of the β_2 AR by terbutaline prevents apoptosis, decreases injury, and promotes recovery of renal function (Nakamura et al., 2003, 2004, 2005, 2010); however, in these studies, excessive activation of the β_2 AR decreased serum

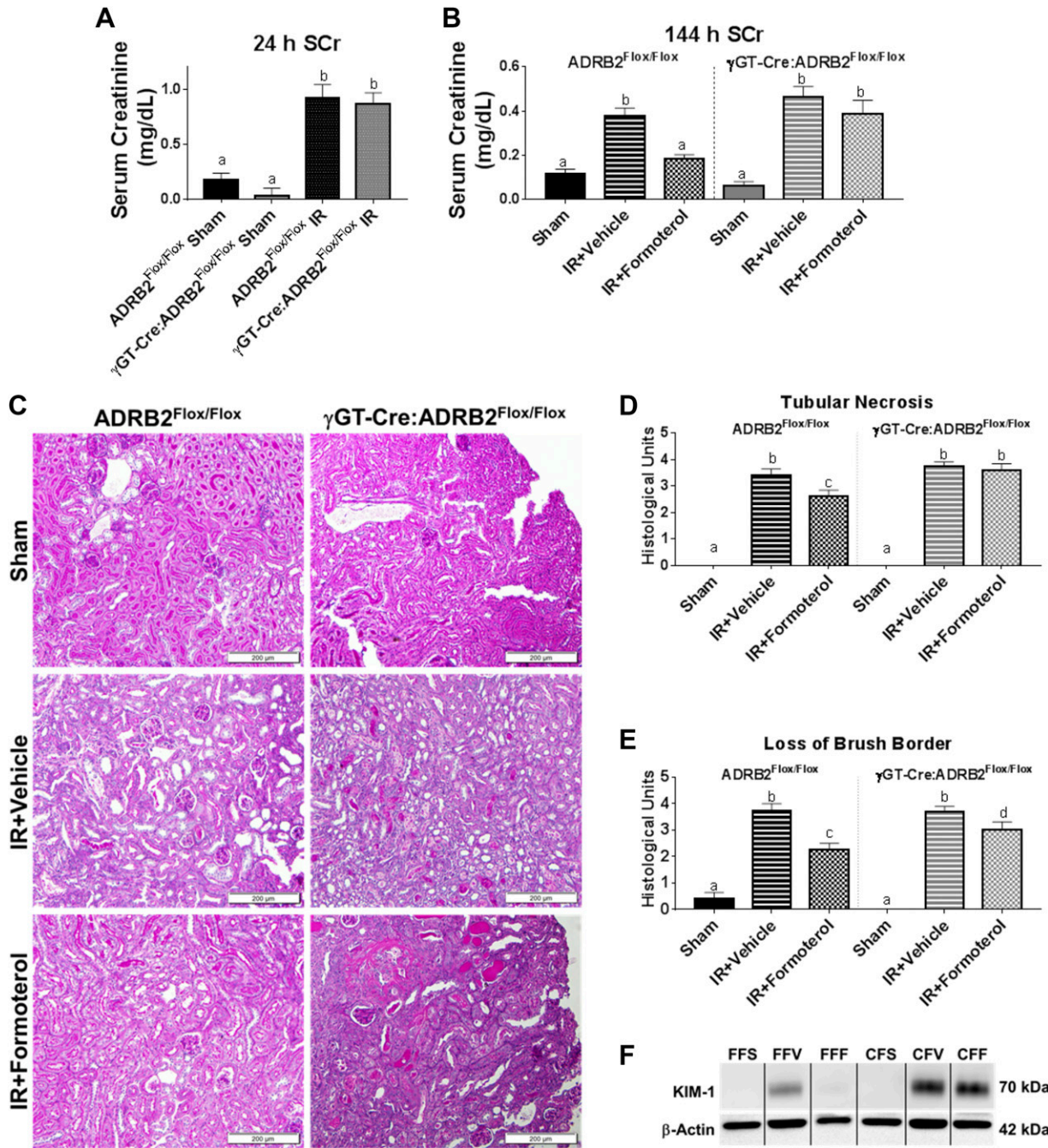


Fig. 2. Proximal tubule deletion of the β_2 AR blocks the effects of formoterol on renal function after AKI. $ADRB2^{Flox/Flox}$ and γ GT-Cre: $ADRB2^{Flox/Flox}$ mice were subjected to sham or renal IRI surgery. Mice were treated with 0.3% DMSO (vehicle) or 0.3 mg/kg formoterol (formoterol) once daily beginning at 24 hours and were euthanized at 144 hours (A and B) Serum creatinine at 24 (A) and 144 hours (B) after IR. (C) PAS-stained kidney sections. (D and E) Semiquantitative scoring of tubular necrosis (D) and loss of brush border (E). (F) Representative blot of renal cortical KIM-1 at 144 h after IRI. FFS- $ADRB2^{Flox/Flox}$ sham, FFV- $ADRB2^{Flox/Flox}$ IR+0.3% DMSO, FFF- $ADRB2^{Flox/Flox}$ IR+0.3 mg/kg formoterol, CFS- γ GT-Cre: $ADRB2^{Flox/Flox}$ Sham, CFV- γ GT-Cre: $ADRB2^{Flox/Flox}$ IR+0.3% DMSO, CFF- γ GT-Cre: $ADRB2^{Flox/Flox}$ IR + formoterol. Mean \pm S.E.M. $n = 4-9$. Different letters denote $P < 0.05$, two-way analysis of variance (ANOVA) with Fisher's least significant difference (LSD) test.

creatinine (Nakamura et al., 2007). We have shown that selective and limited activation of the β_2 AR by formoterol accelerates recovery from IR-induced AKI (Jesinkey et al., 2014), and in these studies, we have shown that formoterol does so by activating RPTC β_2 AR. In mice lacking RPTC β_2 AR, formoterol treatment failed to rescue serum creatinine, KIM-1 protein expression, and tubular necrosis and only partially restored brush border after IR.

Previous studies have identified RPTCs as central mediators of AKI. After injury, RPTC upregulate inflammatory mediators, such as toll-like receptors, chemokines, and cytokines (Bonventre, 2014). Selective injury of RPTCs using inducible diphtheria toxin can cause AKI in mice and subsequent renal fibrosis, and the degree of RPTC death correlated with AKI severity (Grgic et al., 2012; Takaori et al., 2016). Conversely, increasing RPTC proliferation by treatment with

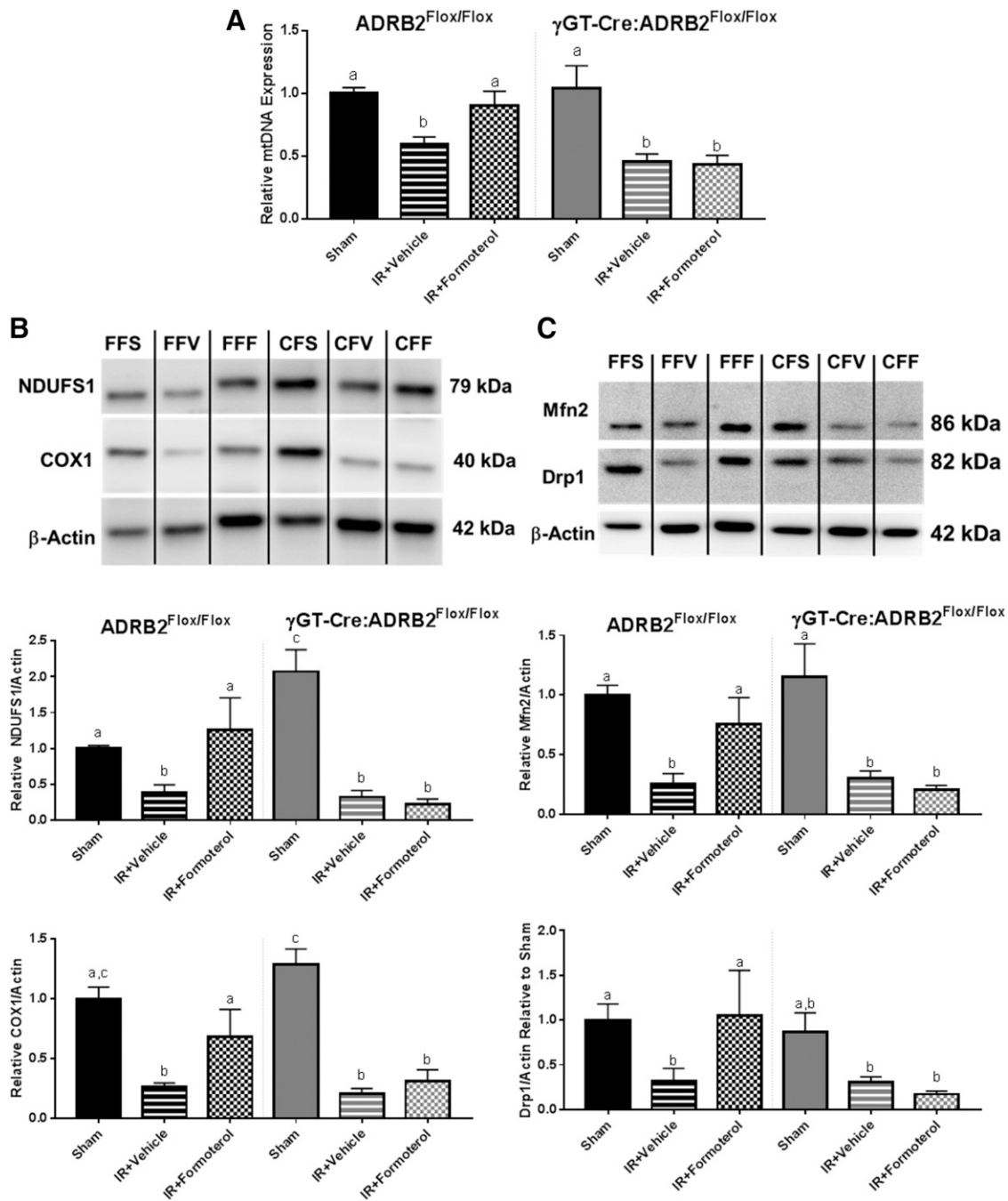


Fig. 3. Proximal tubule cell β_2AR mediates formoterol-induced rescue of mitochondrial homeostasis after IRI-AKI. (A) Mitochondrial DNA copy number. (B) Representative blots and quantification of nuclear-encoded (NDUFS1) and mitochondrial-encoded (COX1) proteins in renal cortex 144 hours after IRI. (C) Representative blots and quantification of mitochondrial dynamic proteins Mfn2 and Drp1 in renal cortex 144 hours after IRI. FFS- $ADRB2^{Flox/Flox}$ sham, FFV- $ADRB2^{Flox/Flox}$ IR + 0.3% DMSO, FFF- $ADRB2^{Flox/Flox}$ IR + 0.3 mg/kg formoterol, CFS- $\gamma GT-Cre:ADRB2^{Flox/Flox}$ sham, CFV- $\gamma GT-Cre:ADRB2^{Flox/Flox}$ IR + 0.3% DMSO, CFF- $\gamma GT-Cre:ADRB2^{Flox/Flox}$ IR + 0.3 mg/kg formoterol. Mean \pm S.E.M. $n = 4-9$. Different letters denote $P < 0.05$, Two-way analysis of variance (ANOVA) with Fisher's least significant difference (LSD) test.

growth factors was associated with enhanced recovery from AKI in rats and dogs (Humes et al., 1989; Miller et al., 1994a,b; Petrincic et al., 1996). In this study, we have shown that mice lacking RPTC β_2AR fail to recover renal function after treatment with formoterol. These data underscore the importance of RPTCs and competent RPTC signaling in the pharmacologic treatment of AKI.

The $\gamma GT1$ promoter has been used by several groups to generate proximal tubule-specific deletions of target genes

(Iwano et al., 2002; Chen et al., 2012; Inoue et al., 2012; Tiwari et al., 2013; Han et al., 2016; Zhou et al., 2018). Although $\gamma GT1$ can also be expressed in hepatocytes, it is expressed in much higher levels in proximal tubule cells (Hanigan et al., 2015), and $\gamma GT-Cre$ mice primarily express Cre in the kidney (Iwano et al., 2002). Further work showed that $\gamma GT-Cre$ deletes floxed genes in only proximal tubule cells (Chen et al., 2012; Inoue et al., 2012; Tiwari et al., 2013; Han et al., 2016). Although γGT isoforms can be expressed by other cell

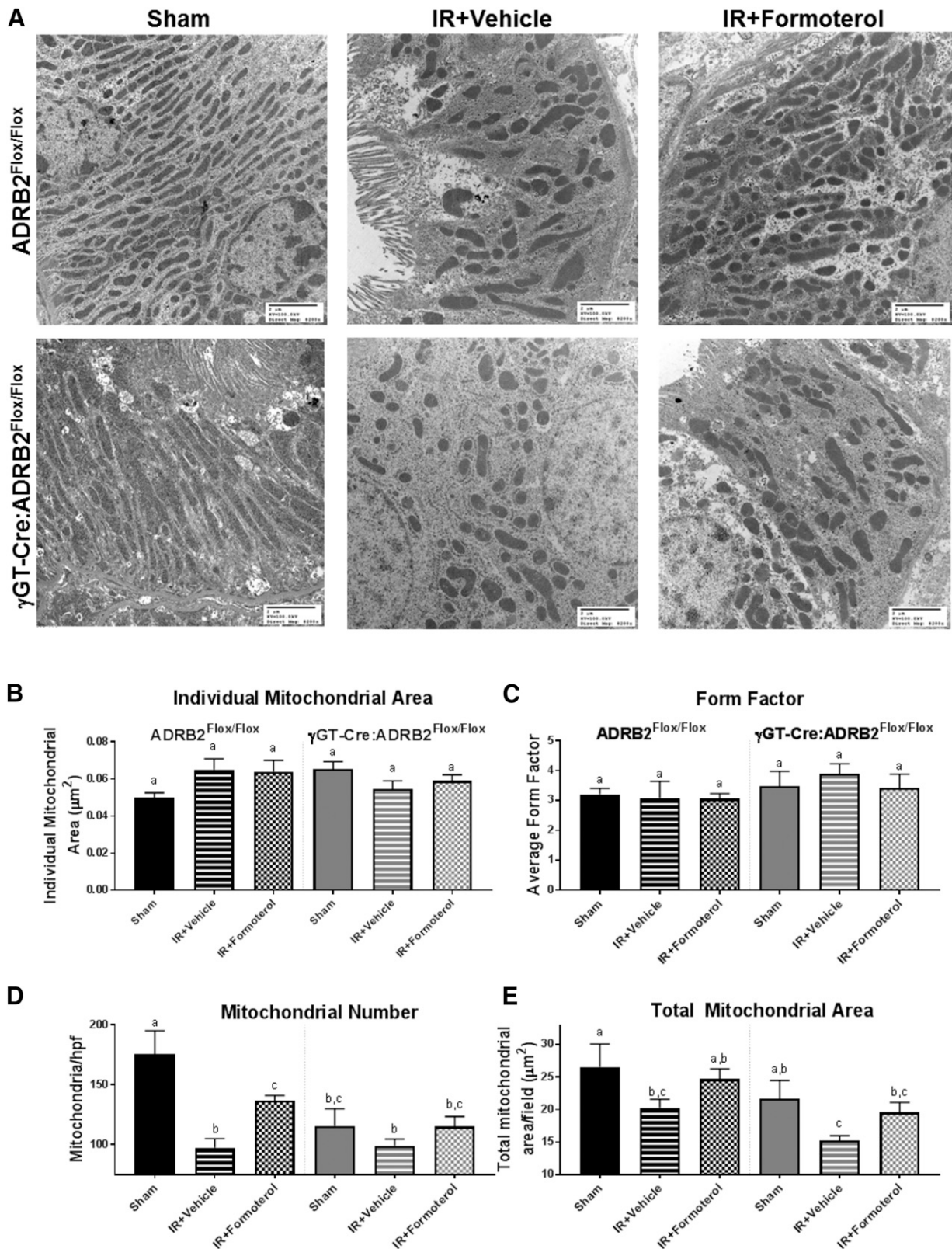


Fig. 4. The role of proximal tubule cell β_2 adrenergic receptor on mitochondrial content and morphology in renal cortex. (A) Representative electron micrographs of ADRB2^{Flox/Flox} and γ GT-Cre:ADRB2^{Flox/Flox} subjected to sham or IRI surgery, followed by treatment with 0.3% DMSO or 0.3 mg/kg formoterol daily for 144 hours. (B) Quantification of individual mitochondrial area. (C) Quantification of average mitochondrial form factor. (D) Quantification of mitochondria per field. (E) Quantification of total mitochondrial area per field. All images were acquired at $\times 8200$ magnification with at least five fields per animal. $n = 3-8$. Mean \pm S.E.M. Different letters indicate $P < 0.05$, Two-way analysis of variance (ANOVA) with Fisher's least significant difference (LSD) test.

types (Hanigan et al., 2015), particularly in pathologic states such as polycystic kidney disease (Starremans et al., 2008) and epithelial-mesenchymal transition (Inoue et al., 2010), the γ GT-Cre mouse generates a proximal tubule-specific deletion of floxed genes without any Cre-dependent effects on renal function.

Despite the numerous pharmacologic modulators for the β_2 AR, there is a paucity of research on its effects in the kidney, particularly in AKI. In our studies, we found no difference in renal function between WT and KO mice at 24 hours, suggesting that proximal tubule deletion of the β_2 AR does not impact renal function in terms of the initial injury after IR. Furthermore, we saw no difference in the renal function or histopathology of sham-operated $ADRB2^{Flox/Flox}$ and γ GT-Cre: $ADRB2^{Flox/Flox}$ mice. Previous work has shown that although increased β_2 AR expression enhances glomerular filtration rate (Nakamura et al., 2004), inhibition of the β_2 AR does not affect renal function but does sensitize animals to septic AKI (Nakamura et al., 2009). Other groups have shown that whole-body deletion of the β_1 AR and β_2 AR reduces baseline renin levels but has a normal response to stimulation (Chen et al., 2010; Neubauer et al., 2011). Additionally, deletion of β_2 AR decreases the activity of a collecting duct-macrophage axis after transverse aortic constriction, although renal function was not measured in these experiments (Fujiu et al., 2017). These effects on renal signaling likely are effects of β_2 AR inhibition on cell types other than the proximal tubule cell.

Proper balance of MB, fission, and fusion is important for recovery from AKI. Deletion of PGC-1 α , a key transcriptional regulator of MB, worsens RPTC injury, whereas its over-expression promotes MB to accelerate recovery (Tran et al., 2011, 2016). Pharmacologic induction of MB accelerates the recovery of mitochondrial and renal function after AKI (Jesinkey et al., 2014; Collier et al., 2016; Collier and Schnellmann, 2017). Formoterol-treated mice lacking the β_2 AR in RPTC failed to recover mtDNA copy number, mitochondrial protein expression, and mitochondrial number and area after AKI. In addition, sham-operated KO mice had fewer total mitochondria without a decrease in mtDNA copy number and elevated NDUF51 protein expression. These data provide evidence that the β_2 AR plays a role in RPTC mitochondrial homeostasis in healthy mice. Whereas previous studies have shown that formoterol increases mRNA, protein, and functional markers of mitochondria in the kidney (Wills et al., 2012; Jesinkey et al., 2014), this study shows for the first time that formoterol induces bona fide MB in RPTC.

In addition to MB, mitochondrial fission and fusion are known to play varying roles in AKI. Because Drp1 and Mfn2 are regulated by the PGC-1 α (Soriano et al., 2006; Martin et al., 2014; Dabrowska et al., 2015) and expression of both proteins is restored after formoterol treatment in WT mice, the recovery of Mfn2 and Drp1 is linked to formoterol-induced activation of PGC-1 α . Drp1 is thought to be detrimental after injury by enhancing mitochondrial fragmentation, reactive oxygen species production, and apoptosis (Tang et al., 2013). After AKI, mitochondrial fragmentation is increased in RPTC in a Drp1-dependent manner (Brooks et al., 2009), and decreased Mfn2 expression potentiates this fragmentation (Gall et al., 2012; Tsushida et al., 2018). As such, formoterol activation of β_2 AR and restoration of Drp1 and Mfn2 expression may improve mitochondrial dynamics and contribute to the recovery of mitochondrial function after IRI by affecting

mitochondrial dynamics; however, we observed no changes in mitochondrial form factor or individual mitochondrial area among our treatment groups, suggesting that the effects of formoterol on mitochondrial dynamics are secondary to those of formoterol on MB.

In summary, these data underscore the importance of RPTC mitochondria as a therapeutic target, that β_2 AR regulates renal mitochondrial homeostasis, and that G protein-coupled receptor ligands such as formoterol can induce MB to accelerate recovery from renal function. Since they represent such a large portion of the pharmacopeia, identification of more G protein-coupled receptor ligands that can induce MB in RPTC will provide a greater number of potential therapeutics for AKI.

Acknowledgments

We thank Dr. Zhi Zhong for assistance in initial establishment of the mouse colony.

Author Contributions

Participated in research design: Cameron, Beeson, Schnellmann.
Conducted experiments: Cameron, Gibbs, Miller, Dupre, Megyesi.
Performed data analysis: Cameron, Miller.
Wrote or contributed to the writing of the manuscript: Cameron, Miller, Dupre, Schnellmann.

References

- Bonventre JV (2014) Primary proximal tubule injury leads to epithelial cell cycle arrest, fibrosis, vascular rarefaction, and glomerulosclerosis. *Kidney Int Suppl* (2011) 4:39–44.
- Brooks C, Wei Q, Cho SG, and Dong Z (2009) Regulation of mitochondrial dynamics in acute kidney injury in cell culture and rodent models. *J Clin Invest* 119: 1275–1285.
- Cameron RB, Beeson CC, and Schnellmann RG (2017) Structural and pharmacological basis for the induction of mitochondrial biogenesis by formoterol but not clenbuterol. *Sci Rep* 7:10578.
- Chen J, Chen JK, Nagai K, Plieth D, Tan M, Lee TC, Threadgill DW, Neilson EG, and Harris RC (2012) EGFR signaling promotes TGF β -dependent renal fibrosis. *J Am Soc Nephrol* 23:215–224.
- Chen L, Kim SM, Eisner C, Oppermann M, Huang Y, Mizel D, Li L, Chen M, Squeira Lopez ML, Weinstein LS, et al. (2010) Stimulation of renin secretion by angiotensin II blockade is G α -dependent. *J Am Soc Nephrol* 21:986–992.
- Chevalier RL (2016) The proximal tubule is the primary target of injury and progression of kidney disease: role of the glomerulotubular junction. *Am J Physiol Renal Physiol* 311:F145–F161.
- Collier JB and Schnellmann RG (2017) Extracellular signal-regulated kinase 1/2 regulates mouse kidney injury molecule-1 expression physiologically and following ischemic and septic renal injury. *J Pharmacol Exp Ther* 363:419–427.
- Collier JB, Whitaker RM, Eblen ST, and Schnellmann RG (2016) Rapid renal regulation of peroxisome proliferator-activated receptor γ coactivator-1 α by extracellular signal-regulated kinase 1/2 in physiological and pathological conditions. *J Biol Chem* 291:26850–26859.
- Dabrowska A, Venero JL, Iwasawa R, Hankir MK, Rahman S, Boobis A, and Hajji N (2015) PGC-1 α controls mitochondrial biogenesis and dynamics in lead-induced neurotoxicity. *Aging (Albany NY)* 7:629–647.
- Fujiu K, Shibata M, Nakayama Y, Ogata F, Matsumoto S, Noshita K, Iwami S, Nakae S, Komuro I, Nagai R, et al. (2017) A heart-brain-kidney network controls adaptation to cardiac stress through tissue macrophage activation. *Nat Med* 23: 611–622.
- Funk JA and Schnellmann RG (2012) Persistent disruption of mitochondrial homeostasis after acute kidney injury. *Am J Physiol Renal Physiol* 302:F853–F864.
- Gall JM, Wang Z, Liesa M, Molina A, Havasi A, Schwartz JH, Shirihai O, Borkan SC, and Bonegio RG (2012) Role of mitofusin 2 in the renal stress response. *PLoS One* 7:e31074.
- Grgic I, Campanholle G, Bijol V, Wang C, Sabbiseti VS, Ichimura T, Humphreys BD, and Bonventre JV (2012) Targeted proximal tubule injury triggers interstitial fibrosis and glomerulosclerosis. *Kidney Int* 82:172–183.
- Han X, Yang J, Li L, Huang J, King G, and Quarles LD (2016) Conditional deletion of Fgf1 in the proximal and distal tubule identifies distinct roles in phosphate and calcium transport. *PLoS One* 11:e0147845.
- Hanigan MH, Gillies EM, Wickham N, Wakeham N, and Wirsig-Wiechmann CR (2015) Immunolabeling of gamma-glutamyl transferase 5 in normal human tissues reveals that expression and localization differ from gamma-glutamyl transferase 1. *Histochem Cell Biol* 143:505–515.
- Hinoi E, Gao N, Jung DY, Yadav V, Yoshizawa T, Myers MG Jr, Chua SC Jr, Kim JK, Kaestner KH, and Karsenty G (2008) The sympathetic tone mediates leptin's inhibition of insulin secretion by modulating osteocalcin bioactivity. *J Cell Biol* 183: 1235–1242.
- Hoste EA, Bagshaw SM, Bellomo R, Cely CM, Colman R, Cruz DN, Edeipidis K, Forni LG, Gomersall CD, Govil D, et al. (2015) Epidemiology of acute kidney injury in

- critically ill patients: the multinational AKI-EPI study. *Intensive Care Med* **41**: 1411–1423.
- Humes HD, Cieslinski DA, Coimbra TM, Messina JM, and Galvao C (1989) Epidermal growth factor enhances renal tubule cell regeneration and repair and accelerates the recovery of renal function in posts ischemic acute renal failure. *J Clin Invest* **84**:1757–1761.
- Inoue T, Suzuki H, and Okada H (2012) Targeted expression of a pan-caspase inhibitor in tubular epithelium attenuates interstitial inflammation and fibrogenesis in nephritic but not nephrotic mice. *Kidney Int* **82**:980–989.
- Inoue T, Takenaka T, Hayashi M, Monkawa T, Yoshino J, Shimoda K, Neilson EG, Suzuki H, and Okada H (2010) Fibroblast expression of an I κ B dominant-negative transgene attenuates renal fibrosis. *J Am Soc Nephrol* **21**:2047–2052.
- Iwano M, Plieth D, Danoff TM, Xue C, Okada H, and Neilson EG (2002) Evidence that fibroblasts derive from epithelium during tissue fibrosis. *J Clin Invest* **110**:341–350.
- Jang HR and Rabb H (2015) Immune cells in experimental acute kidney injury. *Nat Rev Nephrol* **11**:88–101.
- Jesinkey SR, Funk JA, Stallons LJ, Wills LP, Megyesi JK, Beeson CC, and Schnellmann RG (2014) Formoterol restores mitochondrial and renal function after ischemia-reperfusion injury. *J Am Soc Nephrol* **25**:1157–1162.
- Liu BC, Tang TT, Lv LL, and Lan HY (2018) Renal tubule injury: a driving force toward chronic kidney disease. *Kidney Int* **93**:568–579.
- Martin OJ, Lai L, Soundarapandian MM, Leone TC, Zorzano A, Keller MP, Attie AD, Muoio DM, and Kelly DP (2014) A role for peroxisome proliferator-activated receptor γ coactivator-1 in the control of mitochondrial dynamics during postnatal cardiac growth. *Circ Res* **114**:626–636.
- Miller SB, Martin DR, Kissane J, and Hammerman MR (1994a) Hepatocyte growth factor accelerates recovery from acute ischemic renal injury in rats. *Am J Physiol* **266**:F129–F134.
- Miller SB, Martin DR, Kissane J, and Hammerman MR (1994b) Rat models for clinical use of insulin-like growth factor I in acute renal failure. *Am J Physiol* **266**:F949–F956.
- Molitoris BA (2014) Therapeutic translation in acute kidney injury: the epithelial/endothelial axis. *J Clin Invest* **124**:2355–2363.
- Moore PK, Hsu RK, and Liu KD (2018) Management of acute kidney injury: core curriculum 2018. *Am J Kidney Dis* **72**:136–148.
- Nakamura A, Imaizumi A, Niimi R, Yanagawa Y, Kohsaka T, and Johns EJ (2005) Adenoviral delivery of the beta2-adrenoceptor gene in sepsis: a subcutaneous approach in rat for kidney protection. *Clin Sci (Lond)* **109**:503–511.
- Nakamura A, Imaizumi A, Yanagawa Y, Kohsaka T, and Johns EJ (2004) beta(2)-Adrenoceptor activation attenuates endotoxin-induced acute renal failure. *J Am Soc Nephrol* **15**:316–325.
- Nakamura A, Imaizumi A, Yanagawa Y, Niimi R, Kohsaka T, and Johns EJ (2003) Beta2-adrenoceptor activation inhibits Shiga toxin2-induced apoptosis of renal tubular epithelial cells. *Biochem Pharmacol* **66**:343–353.
- Nakamura A, Niimi R, Imaizumi A, and Yanagawa Y (2007) Renal effects of beta2-adrenoceptor agonist and the clinical analysis in children. *Pediatr Res* **61**:129–133.
- Nakamura A, Niimi R, and Yanagawa Y (2009) Renal beta(2)-adrenoceptor modulates the lipopolysaccharide transport system in sepsis-induced acute renal failure. *Inflammation* **32**:12–19.
- Nakamura A, Niimi R, and Yanagawa Y (2010) Protection from sepsis-induced acute renal failure by adenoviral-mediated gene transfer of beta2-adrenoceptor. *Nephrol Dial Transplant* **25**:730–737.
- Nakamura M, Shirai A, Yamazaki O, Satoh N, Suzuki M, Horita S, Yamada H, and Seki G (2014) Roles of renal proximal tubule transport in acid/base balance and blood pressure regulation. *BioMed Res Int* **2014**:504808.
- Neubauer B, Machura K, Schnermann J, and Wagner C (2011) Renin expression in large renal vessels during fetal development depends on functional beta1/beta2-adrenergic receptors. *Am J Physiol Renal Physiol* **301**:F71–F77.
- Petrinec D, Reilly JM, Sicard GA, Lowell JA, Howard TK, Martin DR, Brennan DC, and Miller SB (1996) Insulin-like growth factor-I attenuates delayed graft function in a canine renal autotransplantation model. *Surgery* **120**:221–225; discussion 225–226.
- 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.
- Ralto KM and Parikh SM (2016) Mitochondria in acute kidney injury. *Semin Nephrol* **36**:8–16.
- Sekine M, Monkawa T, Morizane R, Matsuoka K, Taya C, Akita Y, Joh K, Itoh H, Hayashi M, Kikkawa Y, et al. (2012) Selective depletion of mouse kidney proximal straight tubule cells causes acute kidney injury. *Transgenic Res* **21**:51–62.
- Soriano FX, Liesa M, Bach D, Chan DC, Palacín M, and Zorzano A (2006) Evidence for a mitochondrial regulatory pathway defined by peroxisome proliferator-activated receptor-gamma coactivator-1 alpha, estrogen-related receptor-alpha, and mitofusin 2. *Diabetes* **55**:1783–1791.
- Starremans PG, Li X, Finnerty PE, Guo L, Takakura A, Neilson EG, and Zhou J (2008) A mouse model for polycystic kidney disease through a somatic in-frame deletion in the 5' end of Pkd1. *Kidney Int* **73**:1394–1405.
- Susantitaphong P, Cruz DN, Cerda J, Abulfaraj M, Alqahtani F, Koulouridis I, and Jaber BL; Acute Kidney Injury Advisory Group of the American Society of Nephrology (2013) World incidence of AKI: a meta-analysis. *Clin J Am Soc Nephrol* **8**:1482–1493.
- Takaori K, Nakamura J, Yamamoto S, Nakata H, Sato Y, Takase M, Nameta M, Yamamoto T, Economides AN, Kohno K, et al. (2016) Severity and frequency of proximal tubule injury determines renal prognosis. *J Am Soc Nephrol* **27**:2393–2406.
- Tang WX, Wu WH, Qiu HY, Bo H, and Huang SM (2013) Amelioration of rhabdomyolysis-induced renal mitochondrial injury and apoptosis through suppression of Drp-1 translocation. *J Nephrol* **26**:1073–1082.
- Tiwari S, Singh RS, Li L, Tsukerman S, Godbole M, Pandey G, and Ecelbarger CM (2013) Deletion of the insulin receptor in the proximal tubule promotes hyperglycemia. *J Am Soc Nephrol* **24**:1209–1214.
- Tran M, Tam D, Bardia A, Bhasin M, Rowe GC, Kher A, Zsengeller ZK, Akhavan-Sharif MR, Khankin EV, Saintgeniez M, et al. (2011) PGC-1 α promotes recovery after acute kidney injury during systemic inflammation in mice. *J Clin Invest* **121**:4003–4014.
- Tran MT, Zsengeller ZK, Berg AH, Khankin EV, Bhasin MK, Kim W, Clish CB, Stillman IE, Karumanchi SA, Rhee EP, et al. (2016) PGC1 α drives NAD biosynthesis linking oxidative metabolism to renal protection. *Nature* **531**:528–532.
- Tsushida K, Tanabe K, Masuda K, Tanimura S, Miyake H, Arata Y, Sugiyama H, and Wada J (2018) Estrogen-related receptor α is essential for maintaining mitochondrial integrity in cisplatin-induced acute kidney injury. *Biochem Biophys Res Commun* **498**:918–924.
- Wills LP, Trager RE, Beeson GC, Lindsey CC, Peterson YK, Beeson CC, and Schnellmann RG (2012) The β 2-adrenoceptor agonist formoterol stimulates mitochondrial biogenesis. *J Pharmacol Exp Ther* **342**:106–118.
- Zhou T, Luo M, Cai W, Zhou S, Feng D, Xu C, and Wang H (2018) Runx-related transcription factor 1 (RUNX1) promotes TGF- β -induced renal tubular epithelial-to-mesenchymal transition (EMT) and renal fibrosis through the PI3K subunit p110 δ . *EBioMedicine* **31**:217–225.

Address correspondence to: Rick G. Schnellmann, College of Pharmacy, University of Arizona, Drachman Hall B307B, 1295 N. Martin, Tucson, AZ 85721. E-mail: schnell@pharmacy.arizona.edu

Article Title: Proximal Tubule β_2 Adrenergic Receptor Mediates Formoterol-Induced Recovery of Mitochondrial and Renal Function After Ischemia-Reperfusion Injury

Authors: Robert B. Cameron, Whitney S. Gibbs, Siennah R. Miller, Tess V. Dupre, Judit Megyesi, Craig C. Beeson, Rick G. Schnellmann

Journal Title: Journal of Pharmacology and Experimental Therapeutics

Table of Contents

Supplemental Table 1. Primer Sequences p. 2

Supplemental Table 2. Antibodies p. 2

Supplemental Table 1. Primer Sequences. The set of primer sequences used in these studies.

| Gene | Forward | Reverse |
|-----------------------|----------------------------|--------------------------------|
| ADRB2 ^{Flox} | CCA AAG TTG TTG CAC GTC AC | GCA CAC GCC AAG GAG ATT AT |
| γ GT-Cre | GCT CTT GGG AGA AGT CAT GC | CAT GTT TAG CTG GCC CAA AT |
| ADRB2 | GTA CCG TGC CAC CCA GA | CCC GGG AAT AGA CAA AGA CCA TC |
| ND6 | TCC AAA CAC AAC CAA CAT CC | TTG GCA TTA AAG CCT TCA CC |
| β -Actin | GGG ATG TTT GCT CCA ACC AA | GCG CTT TTG ACT CAG GAT TTA A |

Supplemental Table 2. Antibodies. The set of antibodies used in these studies

| Protein | Manufacturer | Cat. No. | Lot No. | Dilution |
|----------------------|---------------------|-----------------|----------------|-----------------|
| Actin | Santa Cruz | sc-47778 | J2816 | 1:1,000 |
| Mfn2 | Santa Cruz | sc-50331 | F1614 | 1:1,000 |
| Drp1 | Santa Cruz | sc-32898 | F1715 | 1:1,000 |
| NDUFS1 | Abcam | ab96428 | GR70572-10 | 1:1,000 |
| COX1 | Abcam | ab14765 | GR291384-5 | 1:1,000 |
| KIM-1 | R&D Systems | AF1817 | KCA0317041 | 1:1,000 |
| Donkey Anti-Goat IgG | Abcam | ab97110 | GR302038-10 | 1:5,000 |
| Goat Anti-Rabbit IgG | Abcam | ab6721 | GR3204153-1 | 1:2,000 |
| Anti-Mouse IgG | Abcam | ab97046 | GR264912-2 | 1:10,000 |