Proximal Tubule β$_2$ Adrenergic Receptor Mediates Formoterol-Induced Recovery of Mitochondrial and Renal Function After Ischemia-Reperfusion Injury

Robert B. Cameron,[1,2] Whitney S. Gibbs,[1,2] Siennah R. Miller,[2] Tess V. Dupre,[2] Judit Megyesi,[3] Craig C. Beeson,[1] Rick G. Schnellmann[2,4]

Primary Laboratory: Schnellmann Laboratory, University of Arizona

RBC, WSG, CCB- Department of Drug Discovery and Biomedical Sciences, Medical University of South Carolina, Charleston, SC

RBC, WSG, SRM, TVD, RGS- College of Pharmacy, University of Arizona, Tucson, AZ

JM- Department of Medicine, University of Arkansas for Medical Sciences, Little Rock, AR

RGS-Southern Arizona VA Healthcare System, Tucson, AZ

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Corresponding Author: Rick G. Schnellmann

Drachman Hall B307B

1295 N. Martin

Tucson, AZ 85721

schnell@pharmacy.arizona.edu

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Abbreviations:

AKI- acute kidney injury

RPTC- renal proximal tubule cell

MB- mitochondrial biogenesis

β_2AR-beta-2 adrenergic receptor

SCr- serum creatinine

KIM-1- kidney injury marker-1

ETC- electron transport chain

NDUFS1- NADH :ubiquinone oxidoreductase core subunit S1

COX1- cytochrome c oxidase subunit 1

Mfn2- mitofusin 2

Drp1- Dynamin-related protein 1

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Abstract:

Acute kidney injury (AKI) is the rapid loss of renal function following an insult and renal proximal tubule cells (RPTC) are central to the pathogenesis of AKI. The β_2 adrenergic receptor (β_2AR) agonist formoterol accelerates recovery of renal function in mice following ischemia-reperfusion injury (IRI) with associated rescue of mitochondrial proteins. However, the cell type responsible for this recovery remains unknown. The role of RPTC in formoterol-induced recovery of renal function was assessed in a proximal tubule-specific knockout of the β_2AR (γGT-Cre:ADRB2$^{\text{Flox/Flox}}$). These mice and wild-type controls (ADRB2$^{\text{Flox/Flox}}$) were subjected to renal IRI, followed by once daily dosing of formoterol beginning 24 h post-IRI and euthanized at 144 h. Compared to ADRB2$^{\text{Flox/Flox}}$ mice, γGT-Cre:ADRB2$^{\text{Flox/Flox}}$ mice had decreased renal cortical mRNA expression of the β_2AR. After IRI, formoterol treatment restored renal function in ADRB2$^{\text{Flox/Flox}}$ but not γGT-Cre:ADRB2$^{\text{Flox/Flox}}$ mice as measured by serum creatinine, histopathology, and KIM-1 expression. Formoterol-treated ADRB2$^{\text{Flox/Flox}}$ mice exhibited recovery of mitochondrial proteins and DNA copy number, while γGT-Cre:ADRB2$^{\text{Flox/Flox}}$ mice treated with formoterol did not. Analysis of mitochondrial morphology by transmission electron microscopy demonstrated that formoterol increased mitochondrial number and density in ADRB2$^{\text{Flox/Flox}}$ mice but not in γGT-Cre:ADRB2$^{\text{Flox/Flox}}$ mice. These data demonstrate that proximal tubule β_2AR regulates renal mitochondrial homeostasis. Formoterol accelerates the recovery of renal function following AKI by activating proximal tubule β_2AR to induce mitochondrial biogenesis and demonstrates the overall requirement of RPTC in renal recovery.

Introduction

Acute kidney injury (AKI) is a rapid loss of renal function that occurs in over 20% of hospitalized patients and has a mortality rate of 25%.(Hoste et al., 2015; Susantitaphong et al., 2013) 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 (RPTC) are a highly oxidative and regenerative cell type that plays a central role in the pathogenesis of AKI.(Ralto and Parikh, 2016; Sekine et al., 2012) RPTC exhibit mitochondrial fragmentation and dysfunction with persistent suppression of mitochondrial biogenesis (MB) after AKI.(Brooks et al., 2009; Funk and Schnellmann, 2012; Tran et al., 2011) Transgenic mouse models have shown that decreased MB worsens AKI, while 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 FDA-approved β_2 adrenergic receptor (β_2AR) 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) Recently, we elucidated the mechanism of formoterol-induced MB in RPTC.(Cameron et al., 2017) Formoterol binding to the β_2AR results in the release of G$\beta\gamma$ heterodimer, the activation of Akt, the phosphorylation of eNOS, and increased soluble guanylyl cyclase activity and cGMP. This pathway increased PGC-1α, the master regulator of MB,(Puigserver et al., 1998) with concomitant induction of MB.

Because the β_2AR is ubiquitously expressed (e.g. T cells, macrophages, neutrophils, endothelial cells and RPTC), it is not clear which cell(s) are responsible for formoterol induced MB and recovery of renal function following IR-induced AKI. The goal of this study was to determine the specific role of

RPTC β_2AR in AKI and formoterol-induced recovery of mitochondrial and renal function using a mouse with proximal tubule specific deletion of the β_2AR.(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 γGT-Cre mice,(Iwano et al., 2002) a generous gift from Dr. Leslie Gewin, to generate γGT-Cre:ADRB2$^{Flox/Flox}$ mice. These mice were generated on a C57Bl/6 background, and male 8-10 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 (Sigma-Aldrich F9552) or vehicle (0.3% DMSO in normal saline) *via* intraperitoneal injection. Blood was collected by retro-orbital bleeding puncture and serum creatinine (SCr) was determined using the Creatinine Enzymatic Reagent Assay kit (Diazyme) according to 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 reversed transcribed using the iScript Advanced cDNA Synthesis Kit (BioRad, Hercules, CA) and was added to a real-time SYBR green quantitative polymerase chain reaction master mix (BioRad). Changes in gene expression were calculated based on the Δ-Δ threshold cycle method. Primers are reported in Supplemental Methods.

Mouse tail tips were lysed using DirectPCR Lysis reagent (Viagen). Genomic DNA was amplified using Promega 2X PCR Master Mix 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 polymerase chain reaction master mix (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, St. Louis, MO). Following 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 (NIH, Bethesda, MD) software. Antibodies are reported in Supplemental Methods.

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 5–6 microns from animals at 144 hours after I/R or sham surgery were stained with hematoxylin and eosin and PAS, 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 morphological 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 with a T-test where appropriate. The criterion for statistical differences was p<0.05 for all comparisons.

Results

RPTC-specific deletion of the β_2AR in mice was achieved by breeding γGT-Cre mice with ADRB2$^{Flox/Flox}$ (WT) mice to create a γGT-Cre:ADRB2$^{Flox/Flox}$ mouse (KO) (Figure 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 bands), 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 using qPCR using renal cortical DNA. Expression of ADRB2 was assessed by RT-qPCR using renal cortical mRNA. Consistent with loss of β_2AR in RPTC, KO mice had an 80% reduction in renal cortical ADRB2 DNA and mRNA expression and increased γGT-Cre DNA expression relative to WT mice (Figures 1B-D).

The role of RPTC β_2AR 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 h and then daily for 144 h. WT and KO mice had similar increases in SCr at 24 h, indicating no difference in initial injury (Figure 2A). As previously described,(Jesinkey et al., 2014) WT mice treated with formoterol exhibited recovery from AKI at 144 h as measured by decreases in SCr and renal cortical KIM-1 protein (Figures 2B, 2F). In contrast, KO mice treated with formoterol did not exhibit decreases in SCr and renal cortical KIM-1 at 144 h. These findings were confirmed by histopathology in that formoterol-treated WT mice had less necrosis than vehicle-treated animals, while formoterol failed to decrease necrosis in KO mice at 144 h (Figure 2C-D). Together, these findings provide evidence that following AKI, formoterol exerts its effects on renal recovery by activating the RPTC β_2AR.

RPTC have high mitochondrial content to maintain proper solute transport across the tubular lumen.(Chevalier, 2016; Nakamura et al., 2014; Ralto and Parikh, 2016) Following AKI, MB 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_2AR on mitochondrial content

were assessed by measuring mtDNA and mitochondrial proteins using qPCR and immunoblot analysis,

respectively. Formoterol restored mtDNA copy number in WT but not KO mice after IRI (Figure 3A).

Similarly, KO mice subjected to IRI and treated with formoterol demonstrated no recovery of nuclear-

encoded NDUFS1 and the mitochondrial-encoded COX1, electron transport chain (ETC) proteins and

markers of MB (Figure 3B). Thus, activation of RPTC β_2AR by formoterol rescues markers of MB

following AKI. Interestingly, KO shams had elevated expression of NDUFS1, which suggests that the

β_2AR may regulate mitochondrial homeostasis in healthy RPTC.

In addition to the restoration of ETC proteins, formoterol restored mitochondrial fission and

fusion proteins Drp1 and Mfn2, respectively, in WT but not KO mice (Figure 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 h (Figure 4A-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 (Figure 4D-E). Sham-operated KO mice had fewer

mitochondria, providing evidence that RPTC β_2AR regulates mitochondrial homeostasis under

physiological conditions. Together, these data indicate that formoterol activates RPTC β_2AR to induce

MB and accelerate recovery of renal function following AKI.

Discussion.

We demonstrated that RPTC β_2AR is responsible for the effects of formoterol on renal function

following AKI. Previous studies in septic AKI demonstrated that overexpression or activation of the

β_2AR by terbutaline prevent apoptosis, decrease injury, and promote recovery of renal

function.(Nakamura et al., 2005; Nakamura et al., 2004; Nakamura et al., 2003; Nakamura et al., 2010)

However, in these studies, excessive activation of the β_2AR decreased serum creatinine.(Nakamura et al.,

2007) We have shown that selective and limited activation of the β_2AR 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_2AR. In mice lacking RPTC β_2AR, 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 RPTC as central mediators of AKI. Following injury, RPTC

upregulate inflammatory mediators such as toll-like receptors, chemokines, and cytokines.(Bonventre,

2014) Selective injury of RPTC using inducible diphtheria toxin was sufficient to 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 growth factors was

associated with enhanced recovery from AKI in rats and dogs.(Humes et al., 1989; Miller et al., 1994a; b;

Petrinec et al., 1996) In this study, we show that mice lacking RPTC β_2AR fail to recover renal function

following treatment with formoterol. These data underscore the importance of RPTC and competent

RPTC signaling in the pharmacologic treatment of AKI.

The γGT1 promoter has been used by several groups to generate proximal tubule-specific

deletions of target genes.(Chen et al., 2012; Han et al., 2016; Inoue et al., 2012; Iwano et al., 2002; Tiwari

et al., 2013; Zhou et al., 2018) Although γGT1 can also be expressed in hepatocytes, it is expressed in

much higher levels in proximal tubule cells,(Hanigan et al., 2015) and γGT-Cre mice primarily express

Cre in the kidney.(Iwano et al., 2002) Further work showed that γGT-Cre only deletes floxed genes in

proximal tubule cells.(Chen et al., 2012; Han et al., 2016; Inoue et al., 2012; Tiwari et al., 2013) While

γGT isoforms can be expressed by other cell 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 γGT-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_2AR, 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 at 24 h between WT and KO mice, suggesting that proximal tubule deletion of the β_2AR 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$^{\text{Flox/Flox}}$ and γGT-Cre:ADRB2$^{\text{Flox/Flox}}$ mice. Previous work has shown that while increased β_2AR expression enhances glomerular filtration rate,(Nakamura et al., 2004) inhibition of the β_2AR 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_1AR and β_2AR reduces baseline renin levels but have a normal response to stimulation.(Chen et al., 2010; Neubauer et al., 2011) Additionally, deletion of β_2AR decreases the activity of a collecting duct-macrophage axis following transverse aortic constriction, although renal function was not measured in these experiments.(Fujiu et al., 2017) These effects on renal signaling are likely due to the effects of β_2AR inhibition on other cell types than the proximal tubule cell.

Proper balance of MB, fission, and fusion is important for recovery from AKI. Deletion of PGC-1α, a key transcriptional regulator of MB, worsens RPTC injury while its overexpression promotes MB to accelerate recovery.(Tran et al., 2011; Tran et al., 2016) Pharmacologic induction of MB accelerates recovery of mitochondrial and renal function following AKI.(Collier and Schnellmann, 2017; Collier et al., 2016; Jesinkey et al., 2014) Formoterol-treated mice lacking the β_2AR in RPTC failed to recover mtDNA copy number, mitochondrial protein expression, and mitochondrial number and area following AKI. In addition, sham-operated KO mice had fewer total mitochondria without a decrease in mtDNA copy number and elevated NDUFS1 protein expression. These data provide evidence that the β_2AR plays a role in RPTC mitochondrial homeostasis in healthy mice. While previous studies have shown that formoterol increases mRNA, protein, and functional markers of mitochondria in the kidney,(Jesinkey et

al., 2014; Wills et al., 2012) 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-1α,(Dabrowska et al., 2015; Martin et al., 2014; Soriano et al., 2006) and expression of both proteins is restored following formoterol-treatment in WT mice, the recovery of Mfn2 and Drp1 is linked to formoterol-induced activation of PGC-1α. Drp1 is thought to be detrimental following injury by enhancing mitochondrial fragmentation, reactive oxygen species production, and apoptosis.(Tang et al., 2013) Following 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_2AR and restoration of Drp1 and Mfn2 expression may improve mitochondrial dynamics and contribute to 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_2AR regulates renal mitochondrial homeostasis, and that GPCR 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 GPCR ligands that can induce MB in RPTC will provide a greater number of potential therapeutics for AKI.

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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

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Footnotes.

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Person to Receive Reprint Requests:

JPET # 252833

Dr. Rick G. Schnellmann

1295 N. Martin

Drachman B307B

Tucson, AZ 85721

schnell@pharmacy.arizona.edu

Figure Legends.

Figure 1. γGT-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 gGT-Cre (lane 2); a mouse γGT-Cre:ADRB2$^{+/+}$ homozygous for ADRB2^{+} (lane 3) and

expressing gGT-Cre (lane 4); and a γGT-Cre:ADRB2$^{Flox/Flox}$ mouse homozygous for ADRB2^{Flox} (lane 5)

and expressing gGT-Cre (lane 6). B. RT-PCR of ADRB2 mRNA in ADRB2$^{Flox/Flox}$ and γGT-

Cre:ADRB2$^{Flox/Flox}$ mice. C, D. PCR of ADRB2 (C) and γGT-Cre (D) DNA. All samples are from renal

cortex. Mean$\pm$SEM. N=4-5, *-p<0.05, Student's T-test.

Figure 2. Proximal tubule deletion of the β_2 adrenergic receptor blocks the effects of formoterol on renal

function following AKI. ADRB2$^{Flox/Flox}$ and γGT-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 h and were euthanized at 144 h. A, B.) Serum creatinine at 24 h (A) and 144 h (B)

following IR. C.) PAS stained kidney sections. D,E.) Semi-quantitative scoring of tubular necrosis (D).

E.) Representative blot of renal cortical KIM-1 at 144 h following 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-γGT-

Cre:ADRB2$^{Flox/Flox}$ Sham, CFV- γGT-Cre:ADRB2$^{Flox/Flox}$ IR+0.3% DMSO, CFF- γGT-Cre:ADRB2$^{Flox/Flox}$

IR+Formoterol. Mean$\pm$SEM. N=4-9. Different letters denote p<0.05, Two-Way ANOVA with Fisher's
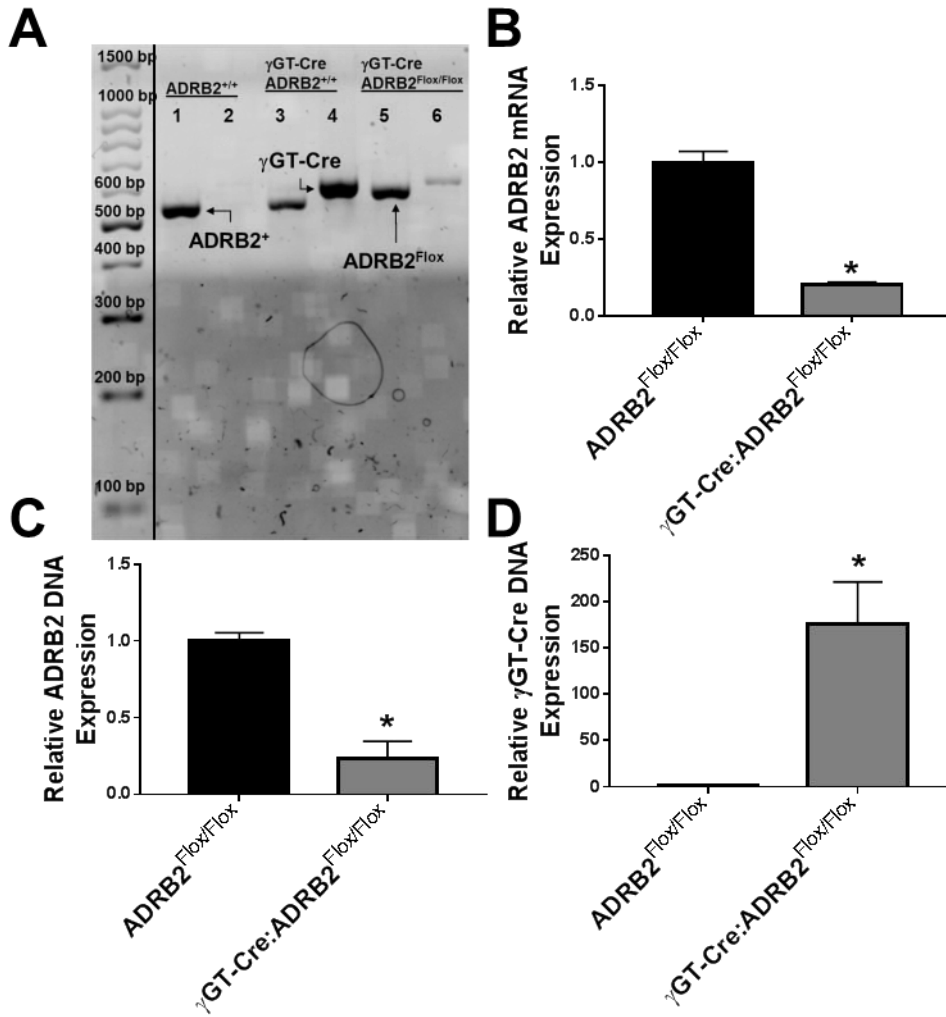
LSD test.

Figure 3. Proximal tubule cell β_2 adrenergic receptor mediates formoterol-induced rescue of

mitochondrial homeostasis following 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 h after IRI. C.) Representative blots and quantification of mitochondrial dynamic

proteins Mfn2 and Drp1 in renal cortex 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-γGT-Cre:ADRB2$^{Flox/Flox}$ sham,

CFV- γGT-Cre:ADRB2$^{Flox/Flox}$ IR+0.3% DMSO, CFF- γGT-Cre:ADRB2$^{Flox/Flox}$ IR+0.3 mg/kg Formoterol.

Mean±SEM. N=4-9. Different letters denote $p<0.05$, Two-Way ANOVA with Fisher's LSD test.

Figure 4. The role of proximal tubule-cell b$_2$ adrenergic receptor on mitochondrial content and morphology in renal cortex. A.) Representative electron micrographs of ADRB2$^{Flox/Flox}$ and γGT-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 h. 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 x8,200 magnification with at least 5 fields per animal. N=3-8. Mean±SEM. Different letters indicate $p<0.05$, Two-Way ANOVA with Fisher's LSD test.
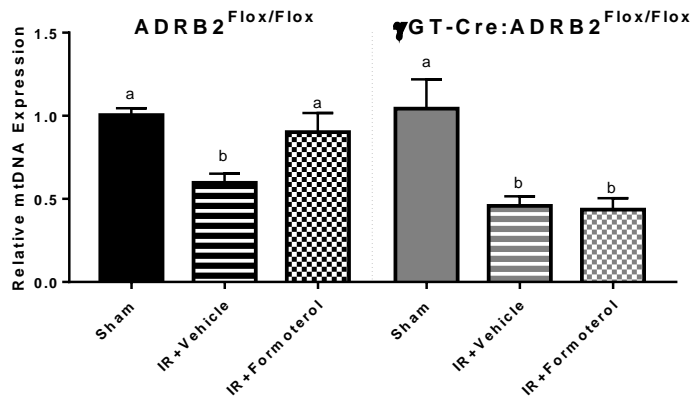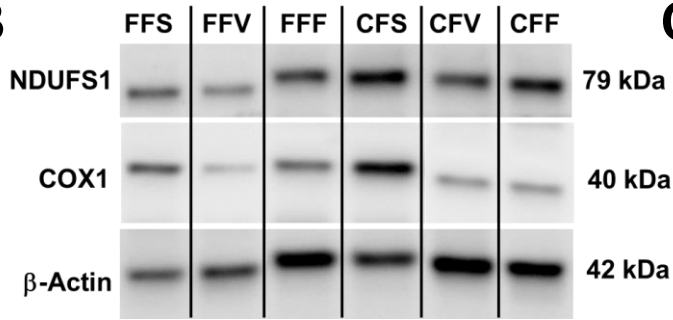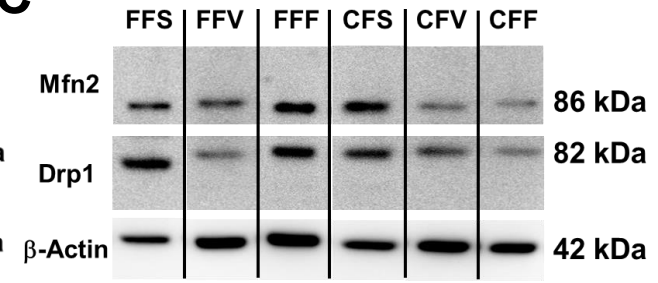
Figure 1.

Figure 2.

Figure 3.

Figure 4.