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The Role of the 5-hydroxytryptamine 1F Receptor in Mitochondrial Biogenesis and Acute Kidney Injury

By

Whitney Sharee Gibbs

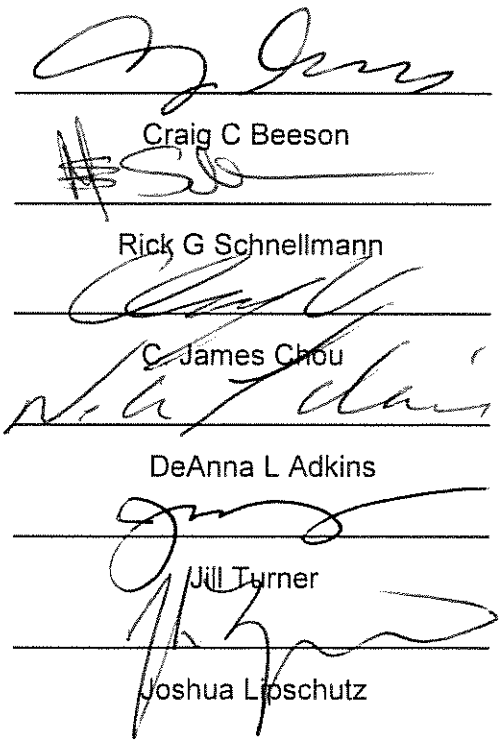
A dissertation submitted to the faculty of the Medical University of South Carolina in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Graduate Studies.

Program in Drug Discovery and Biomedical Sciences

2017

Approved by:

Chairman, Advisory Committee



Dedication

This dissertation is dedicated to my family and friends who have provided unwavering support, love and encouragement throughout each step of my educational journey.

Acknowledgments

I would like to thank Dr. Rick Schnellmann for his guidance and mentoring throughout my graduate career. I also wish to acknowledge my committee members: Dr. Craig Beeson, Dr. James Chou, Dr. DeAnna Adkins, Dr. Joshua Lipschutz and Dr. Jill Turner for their advice and support. I am also extremely thankful for the past and present members of Dr. Schnellmann's laboratory for their training, guidance and technical support.

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List of Abbreviations

5-HT	5-hydroxytryptamine
AIN	Acute interstital nephritis
AKI	Acute kidney injury
AMPK	AMP-activated protein kinase
ATN	Acute tubular necrosis
ATP	Adenosine triphosphate
BNIP3	BCL2 interating protein 3
BUN	Blood urea nitrogen
cAMP	Cyclic adenosine monophosphate
CKD	Chronic kidney disease
CNS	Central nervous system
COX	Cytochrome c oxidase
DOI	1-(2,5-dimethyoxy-4-iodophenyl)-2-aminopropane
Drp1	Dynamin-related protein 1
ERK	Extracellular signal-regulated kinase
ERR	Estrogen related receptor
ESRD	End-stage renal disease
ETC	Electron transport chain
Fis1	Fission 1
FOXO3a	Forkhead box protein O3
FUNDC1	FUN14 domain containig 1
GFR	Glomerular filtration rate
GPCR	G-protein coupled receptors
GTP	Guanine triphosphate

I/R	Ischemia/reperfusion
ICU	Intensive care unit
IL-18	interleukin-18
IL-1β	Interleukin-1β
IMM	Inner mitochondrial membrane
KIM-1	Kidney injury molecule-1
KO	Knock out
MAPK	Mitogen-activated protein kinase
MB	Mitochondrial biogenesis
Mfn	Mitofusin
MOMP	Mitochondrial outer membrane permeabilization
mtDNA	Mitochondrial DNA
ND1	NADH dehydrogenase 1
NDUFB8	NADH dehydrogenase [ubiquinone] 1 beta subcomplex 8
NDUFS1	NADH dehydrogenase Fe-S protein 1
NGAL	Neutrophil gelatinase-associated lipocalin
NOS	Nitric oxide synthase
NRF	Nuclear respiratory factor
NSAIDs	Non-steroidal anti-inflammatory drugs
OCR	Oxygen consumption rates
OMM	Outer mitochondrial membrane
OPA1	Optic atrophy 1
PAS	Periodic acid-schiff
PDE	Phosphodiesterase
PGC-1	Peroxisome proliferator-activated receptor γ coactivator-1

PI3K	Phosphatidylinositol-3-kinases
Pink1	PTEN-induced putative kinase 1
PKA	Protein kinase A
PKG	Protein kinase G
PNS	Peripheral nervous system
PPAR	Peroxisomal proliferator-activated receptor
PRC	PGC-1 related coactivator
PT	Pertussis toxin
RBF	Renal blood flow
RIP140	Receptor-interacting protein 140
ROS	Reactive oxygen species
RPTC	Renal proximal tubule cells
SCr	Serum creatinin
SERT	Serotonin transporter
SIRT	Sirtuin
SOD2	Superoxide dismutase 2
TBHP	Tert-butyl hydroperoxide
TFAM	Mitochondrial transcripton factor A
TNF-α	Tumor necrosis factor-α
UCP	Uncoupling protein
VASP	Vasodilator-stimulated phosphoprotein

ABSTRACT

WHITNEY SHAREE GIBBS. The Role of the 5-hydroxytrptamine 1F Receptor in Mitochondrial Biogenesis and Acute Kidney Injury. (Under the direction of RICK G SCHNELLMANN)

Mitochondrial dysfunction exacerbates cellular injury, impairs energy-dependent repair, and leads to kidney damage and failure following acute kidney injury (AKI). Mitochondrial dysfunction and impaired mitochondrial biogenesis correlate with decreased in peroxisome proliferator-activated receptor coactivator 1-α (PGC-1α), the reported master regulator of mitochondrial biogenesis, and its downstream targets following I/R-induced AKI. Furthermore, recovery of renal function and tubular injury is accelerated following pharmacological induction of mitochondrial biogenesis following I/R-induced AKI. These results suggest that recovery of mitochondrial number and function may be an effective therapeutic strategy in restoring renal function following AKI.

We recently made the novel observation that agonists of 5-HT_{1F} receptor induce mitochondrial biogenesis *in vitro* and *in vivo.* Specifically, following I/R-induced AKI, the 5-HT_{1F} receptor selective agonist LY344864 enhanced the recovery of mitochondrial DNA (mtDNA) copy number and renal function, as indicated by decreased blood urea nitrogen (BUN). These findings demonstrate that 5-HT_{1F} receptor stimulation promotes recovery from AKI and activates mitochondrial biogenesis pathways.

The goal of the first aim was to determine the signaling pathways involved in mediating renal proximal tubule 5-HT_{1F} receptor-induced mitochondrial biogenesis. Using pharmacological approaches, we identified Gβγ heterodimer-dependent activation of Akt/eNOS/cGMP/PKG/PGC-1α and inhibition of c-raf/ERK/FOXO3a pathways as the mechanism responsible for 5-HT_{1F} receptor-induced mitochondrial biogenesis. We also identified Akt as the link between these stimulatory and inhibitory pathways, and that the stimulatory pathway is required for mitochondrial biogenesis. Elucidation of this pathway

may facilitate the development of novel therapeutic approaches to enhance mitochondrial biogenesis for the treatment of diseases characterized by mitochondrial dysfunction.

We then examined the role of the 5-HT_{1F} receptor in renal mitochondrial homeostasis and biogenesis under physiological conditions. To complete this aim, we utilized young (10 weeks) and aged (26 weeks) 5-HT_{1F} receptor knockout (KO) mice. In young 5-HT_{1F} receptor KO mice, we observed increased expression of mtDNA copy number as well as of genes involved in renal mitochondrial biogenesis, oxidative phosphorylation, fission and autophagy compared to wild-type (WT) controls. Aged 5-HT_{1F} receptor KO mice also exhibit increases in renal PGC-1α mRNA expression and mtDNA copy number. Interestingly, we detected a tissue-specific difference in renal cortical mitochondrial homeostasis compared to that of the heart. Specifically, cardiac left ventricular mitochondrial homeostasis markers were initially decreased in the absence of the 5-HT_{1F} receptor KO mice compared to WT mice. However, as the mice aged, these markers returned to WT control levels and this rescue was associated with increased PGC-1α mRNA expression. To determine the potential mechanism responsible for tissue-specific differences in mitochondrial homeostasis markers and the compensatory effect displayed in the renal cortex of the 5-HT_{1F} receptor KO mice, we assessed the gene expression of other 5-HT_1 and 5-HT_2 receptors. Interestingly, in the heart and kidney of 5-HT_{1F} receptor KO mice, there is a tissue-dependent difference in the gene expression of 5-HT_{2A} and 5-HT_{2B} receptors, both of which have been linked to mitochondria. Further work may lead to the identification of compensatory mechanisms that are activated in the absence of the 5-HT_{1F} receptor.

Our final study tested the role of the 5-HT_{1F} receptor in renal mitochondrial biogenesis in AKI and in the recovery of mitochondrial and renal function following I/R-induced AKI. The absence of the 5-HT_{1F} receptor increased tubular injury as measured by KIM-1 and neutrophil gelatinase-associated lipocalin (NGAL) at 24 hr following I/R-induced AKI.

Additionally, the 5-HT_{1F} receptor KO mice exhibited reduced renal recovery at 144 hr following I/R-induced AKI as measured by serum creatinine and BUN levels. Impaired renal function and tubular injury recovery was also associated with a persistent suppression in mitochondrial biogenesis as evidenced by reduced PGC-1α and respiratory chain protein expression at 144 hr following renal I/R injury. Injured 5-HT_{1F} receptor KO mice also displayed sustained depletion in ATP generation and elevated oxidative protein damage at 144 hr. In summary, this study reveals that the 5-HT_{1F} receptor 1) regulates mitochondrial biogenesis and homeostasis under physiological conditions in a tissue-dependent manner, 2) is renal protective in the setting of I/R-induced AKI and 3) promotes the recovery of mitochondrial homeostasis and renal function following I/R injury.

CHAPTER ONE:

Review of acute kidney injury, ischemia/reperfusion, mitochondrial biology and serotonin receptor biology and function

ACUTE KIDNEY INJURY

Definition of Acute Kidney Injury

Acute kidney injury (AKI) is defined broadly as a rapid loss of renal function over hours or days leading to structural damage and disruption in clearance of waste products, electrolyte and fluid balance, and/or urine concentration and output (1). The diagnosis of AKI is traditionally based on a rise in serum creatinine (SCr), indicating altered glomerular filtration rate (GFR) and a fall in urine output. Since outcomes and treatment strategies are directly correlated with severity of diagnostic endpoints, classification systems of AKI were developed to provide consensus and evidence-based guidelines to stratify AKI cases with the primary goal of timely recognition of AKI and outcome prediction of AKI. Specifically, the RIFLE criteria (Risk, Injury, Failure, Loss of function, and End-Stage kidney disease) developed by the Acute Dialysis Quality Initiative (ADQI) workgroup, is perhaps the most comprehensive clinical classification of AKI as it relies on easy clinical applicability, sensitivity and specificity, while accounting for baseline SCr variations and ‘acute-on-chronic’ phenomenon of AKI (2). A similar classification system, referred to as AKIN (Acute Kidney Injury Network), has also been developed for the appropriate stratification of AKI patients (3). An outline of criteria for both RIFLE and AKIN classification systems is provided below (Figure 1.1). These systems combine SCr and urine output to stage patients by injury severity to better predict disease outcomes and mortality (4, 5).

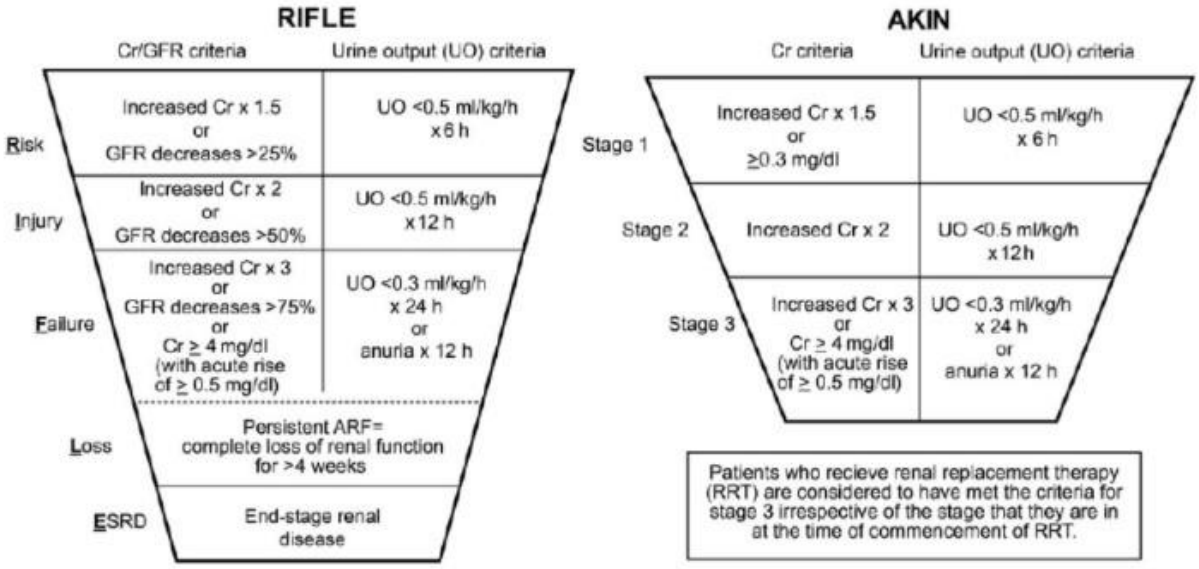

Figure 1.1: RIFLE and AKIN classifications for the staging of AKI. Cr: creatine, ARF: acute renal failure, GFR: glomerular filtration rate, RRT: renal replacement therapy, UO: urine output. Adapted from (6).

Impact of AKI on Global Population

Epidemiology. AKI is now well-established as a common and often under-recognized disorder, which is associated with increased long-term risk of poor outcomes, including death and greater utilization of health resources (7). Observational studies indicate that the incidence of AKI is increasing and patients struggle with the long-term sequelae of AKI (8). In a community based assessment following approximately four million individuals in the United States, the incidence of non dialysis-requiring AKI and dialysis requiring AKI was estimated at 384 and 24 per 10,000 persons-years, respectively (9). The occurrences of AKI is most common among elderly, male and hospitalized patients. A systemic review conducted in developed nations, which included 49 million patients, found that AKI occurred in one in five adults and one in three children hospitalized with acute illness (10). Among critically ill patients in the intensive care unit (ICU), the incidence of AKI rates are as high as 70% (11).

Morbidity and Mortality. Increasing severity of AKI correlates with increased mortality in various clinical settings, such as cardiac surgery (12), major trauma (13), and critical illness (14). AKI-associated mortality occurs at an alarming rate of approximately 24% in adults and 14% in children (10). In-patient mortality is highest among ICU patients requiring renal replacement therapy (RRT), with incidence of mortality approaching 60%.

Although mortality is concurrently declining due to better diagnosis and supportive strategies, patients who survive an acute episode of AKI have an increased risk of morbidity associated with the disease, leading to poor outcomes and long-term mortality. In a retrospect assessment following US veterans who were discharged following acute-care hospitalization, long-term mortality was approximately two times greater of those with AKI compared to those who did not have AKI (15). Among AKI survivors, intermediate and long-term mortality has a strong association with the development of

chronic kidney disease (CKD) and further progression of end-stage renal disease (ESRD) (16), leading to poor quality of life and significant global financial burden. Taken together, given the significant long-term morbidity following AKI, there is an estimated 34 million hospitalizations per year, with $10 billion in healthcare expenses annually in the US (17).The figure below illustrates the relationship between SCr and AKI-associated mortality and healthcare costs (Figure 1.2, 3).

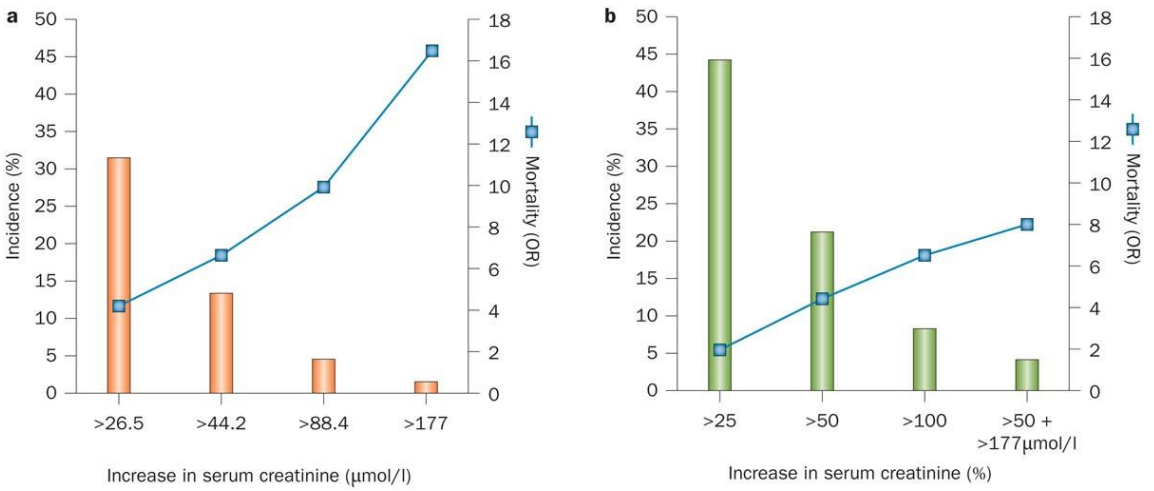

Figure 1.2: Association between severity of AKI and mortality. Orange bars represent incidence percentage as a result of absolute change in SCr levels, blue line graph reflects odds ratio (OR) of mortality in respect to absolute change in SCr levels (a). Green bars represent incidence percentage as a result of changes in SCr levels relative to baseline, blue line graph reflects OR of mortality in respect to adjusted SCr levels (b). Adapted from (18).

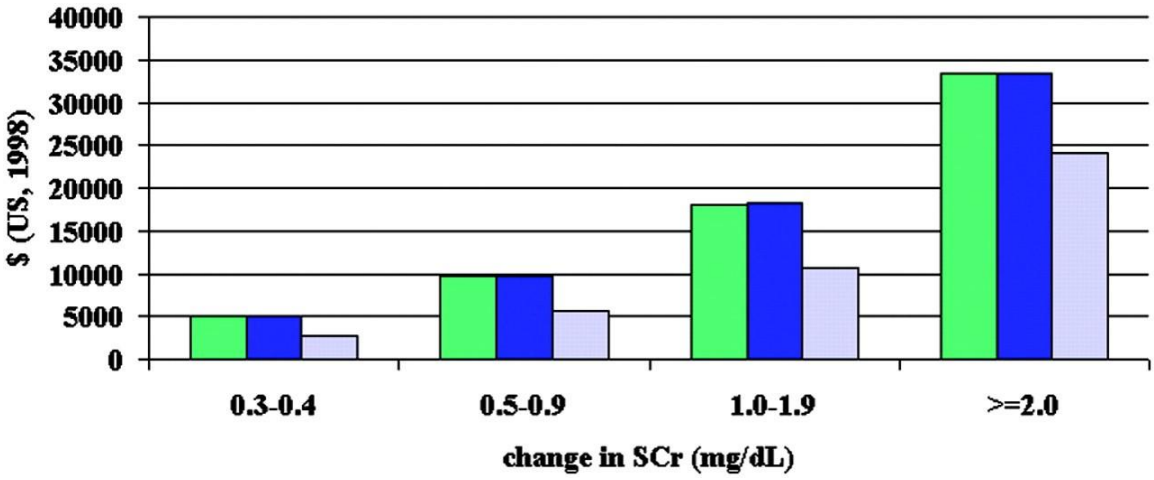

Figure 1.3: Association between severity of AKI and healthcare cost. Green bars represent unadjusted data, blue bars are adjusted for age and gender and grey bars are adjusted for age, gender, diagnosis related-group (DRG) weight and ICD-9-CM codes for cardiovascular, respiratory, malignant, and infectious diseases; n= 1564, 885, 246 and 105 for SCr changes of 0.3 to 0.4, 0.5 to 0.9, 1.0 to 1.9 and ≥ 2.0 mg/dl, respectively. Adapted from (17).

Causes of AKI

AKI is often classified into three categories based on the causes of AKI: prerenal (caused by decreased renal perfusion), intrinsic renal (caused by a process within the kindeys) and postrenal (caused by inadequate drainage of urine distal to the kidneys) (19).

Prerenal AKI. Prerenal AKI is estimated to account for approximately 70% of community-acquired AKI and 40% of hospital-acquired AKI (20, 21). Prerenal AKI is a result of decrease renal blood flow (RBF) and subsequent renal hypoperfusion in the absence of renal parenchyma damage (22). Most common causes of prerenal AKI include cardiac surgery, septic shock and congestive heart failure (21, 23). Prerenal AKI can also occur in the setting of hypovolemia resulting from hemorrhage, vomiting, poor fluid intake and diuresis. Several medications can also cause prerenal AKI, notably, angiotensin-converting enzyme inhibitors, non-steroidal anti-inflammatory drugs (NSAIDs), vasoconstrictors and diuretics (19). It is important to note that prolong or profound prerenal AKI can result in ischemic damage to the kidney and thus inducing renal tubular epithelial cell structural and functional alterations. The extent of these alterations is dependent upon severity and duration of prerenal and ischemic injury.

Intrinsic AKI. Intrinsic AKI is estimated to account for approximately 35-40% of all patients with AKI (24). Intrinsic AKI is classified anatomically by the region of the kidney parenchyma that is damaged; these four structures are the tubules, glomeruli, interstitum and intrarenal vessels. Tubular injury as a result of acute tubular necrosis (ATN) is the most common among hospitalized AKI patients. The development of ATN typically follows exposure to a nephrotoxic compound such as aminoglycoside antibiotics and chemotherapeutic drugs, or an ischemic event, with the latter being the most common.

Ischemia as a result of profound obstruction of blood flow leads to direct injury to tubules, thus prerenal AKI and intrinsic AKI represent a continuum of damage due to a common insult (19).

A less common cause of intrinsic AKI, acute interstitial nephritis (AIN) is most often caused by an allergic reaction to a drug, such as certain classes of antibiotics, penicillins and cephalosporin and NSAIDs. Additionally, patients with autoimmune diseases and systemic infections, such as systemic lupus erythematosus and HIV, have increased susceptibility for the development of AIN (25). Allergic reactions to medications and altered immune systems have been reported to induce immune-mediated tubulointerstitial injury, resulting in diminished tubular function. It is important to note that patients of autoimmune diseases are at risk for glomerulonephritis, inflammation and damage to the glomerular membrane.

Postrenal AKI. Postrenal AKI is relatively uncommon accounting for 20% of community-acquired AKI and <5% of hospital- acquired AKI (26). Postrenal AKI is caused by an acute obstruction of the urinary flow causing waste build up in the kidneys and a subsequent increase in tubular pressure and reduction in GFR. Postrenal AKI occurs more often in elderly male patients with prostatic hypertrophy or prostate cancer (27). Additional causes of postrenal AKI include gynecologic cancers, such as cervical cancer, ureteral stones and precipitation of various substances such as acyclovir (28).

The multifactorial nature of AKI contributes to the complexity of this condition and the unsuccessful attempts to develop therapeutic treatments. Understanding the overwhelming number of pathological contributors and mechanisms of AKI will help lead to the development of drug therapies in the hope of in decreasing the morbidity and mortality associated with AKI.

Since the studies presented herein will address ischemia reperfusion-induced AKI, this review will focus intrinsic AKI with particular emphasis on the role of the proximal tubular epithelial cells.

Pathogenesis of Ischemia/Reperfusion-induced AKI

Ischemia/ reperfusion (I/R)-induced AKI is associated with an unacceptable high incidence and mortality due to the incredible number of patients that are at risk for ischemic AKI, including patients of trauma, major surgery, congestive heart failure, and sepsis. Renal I/R injury is characterized by a sudden blockade of oxygen and nutrient delivery to the kidneys (29). The complex interactions between epithelial, endothelia and inflammatory cells, as well as dysfunctional mitochondria and reactive oxygen species (ROS) substantially contribute to the extension phase of I/R- induced AKI (Figure 1.5).

Endothelial Injury. Endothelial cells are important regulators of vascular tone, blood flow, vascular permeability, leukocyte function, and smooth muscle responsiveness (30). An important feature of renal I/R injury is prominent damage to the endothelium. In turn, vasoconstriction is significantly enhanced as a result of decreased nitric oxide production and enhanced production of vasoactive cytokines, including TNF-α, IL-1β and endothelin, generated as a consequence of increased leukocyte- endothelial adhesion and leukocyte activation following ischemic injury (31-34). In addition, upregulation of chemokines and adhesion molecules in the endothelium results in the infiltration of inflammatory cells like neutrophils, lymphocytes and macrophages from the blood vessels into the interstitum of the kidney (35, 36). These inflammatory cells can produce vasoconstrictors, such as prostaglandins, that have the reputation to worsen blood flow and vascular injury. Recent attention has focused on the impact of endothelial

mitochondria in the role of vasoconstriction and capillary permeability due to altered calcium and ATP homeostasis (37, 38).

Epithelial Injury. Tubular epithelial cell dysfunction and death of one or several tubular segments following I/R renal injury is common in the development of ATN. Specifically, renal proximal tubule cells (RPTC), the most populous cell type in the kidney regulating reabsorption of substances such as glucose and amino acids and the control of acid-base balance by the excretion of bicarbonate and synthesis of ammonia, are particularly sensitive to ischemic injury (39, 40). Under physiological conditions, RPTC attach to the basement membrane and maintain high polarity with both the apical and basolateral membrane. Na^+/K^+-ATPase on the basolateral membrane utilizes ATP to establish the sodium gradient that drives subsequent secondary and tertiary transport processes across both the apical and basolateral membranes (38). After renal I/R injury, transport processes necessary for the reabsorption and secretion of molecules are disrupted due to loss of polarity, marked reduction in cellular ATP content, mislocalization of the Na^+/K^+-ATPase and decline in its activity (41, 42). In addition, mitochondrial dysfunction and cytoskeletal disruptions are also critical pathogenic factors in renal I/R injury (43). With increasing severity, these cellular impairments leads to RPTC apoptosis and necrosis. As a result, detachment of both necrotic and viable cells that have lost basolateral expression of integrin receptors slough into the tubular lumen and form tubular casts, potentiating injury and decline in GFR (41, 44, 45).

Several reports indicate that surviving RPTC restore physiological functions and nephron integrity. The surviving cells dedifferentiate, migrate along the basement membrane, proliferate to restore cell number and then differentiate (19, 44, 46). Figure 1.5 illustrates normal repair of the RPTC following renal I/R injury. While the precise mechanisms of renal repair and recovery remain largely unknown, recent attention has focused on the

role of mitochondria in mediating restoration of tubule function. Proximal tubule epithelial cells are rich in mitochondria relying heavily on oxidative phosphorylation for ATP generation, so, RPTC are especially vulnerable to mitochondrial dysfunction (47). Recent evidence indicates that promoting the repair of mitochondrial structure and function is a promising strategy to facilitate recovery of the damaged kidney. Targeting RPTC mitochondria will be the major focus of this review.

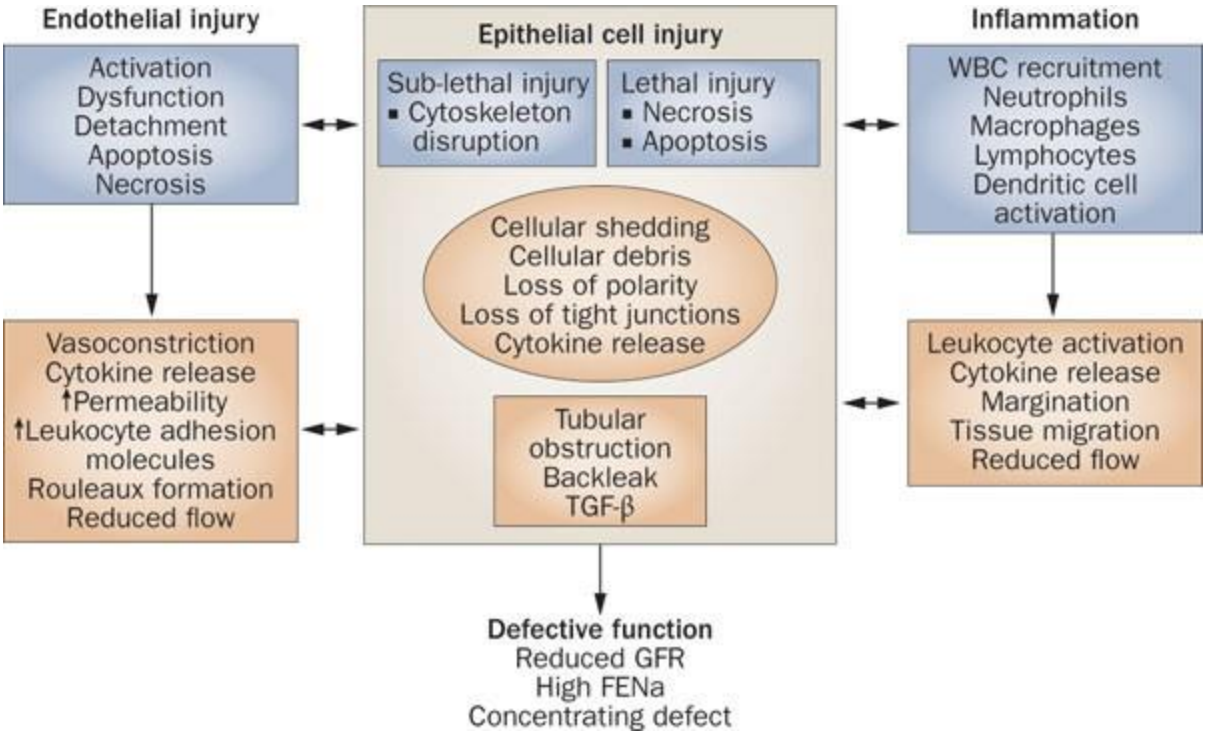

Figure 1.4: The Pathogenesis of Ischemic AKI. Major pathways of ischemic AKI include the complex interaction between endothelial and epithelial cells. These processes are regulated by the activation of inflammatory response, vasoconstriction, alteration in adhesion molecules expression, apoptosis, necrosis and cytoskeleton disruption. Ultimately, reduction in glomerular filtration rate (GFR), fractional excretion of sodium (FENa) and defect in urine concentration. WBC: white blood cells, TGF-β: transforming growth factor β. Adapted from (48).

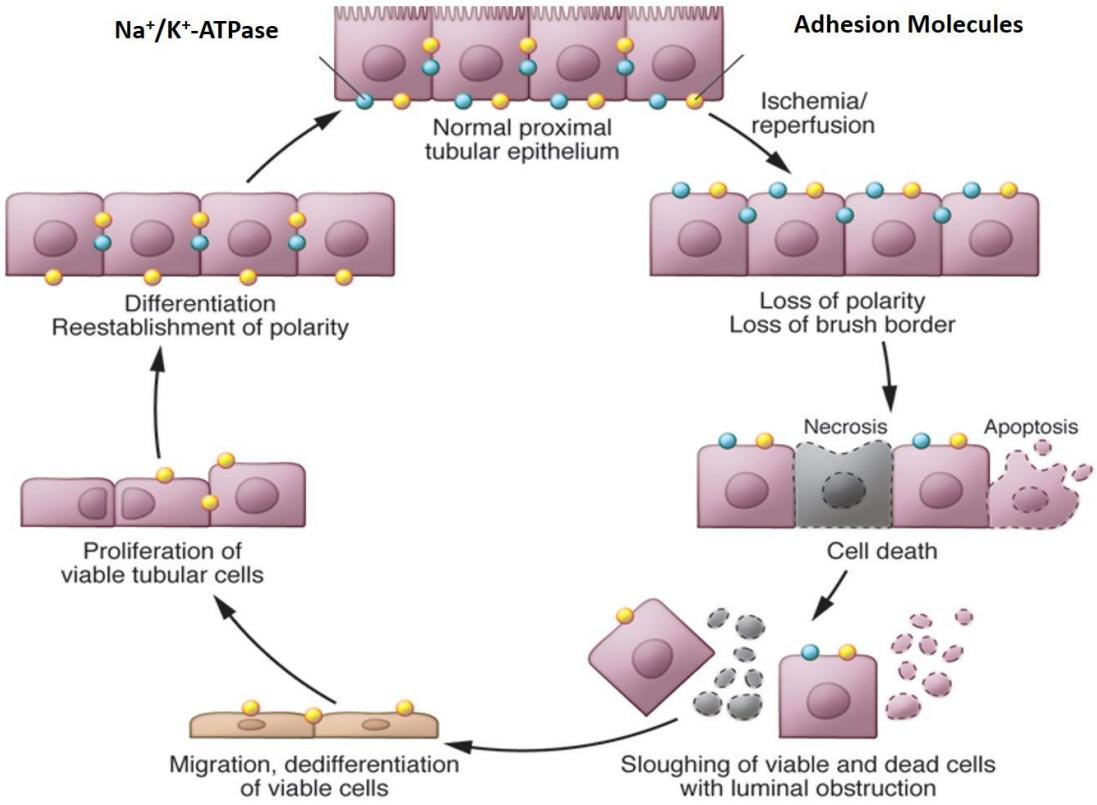

Figure 1.5: Normal Repair in Ischemic AKI. Following ischemic renal injury, renal epithelial cells lose their polarity and brush border with proteins translocated from the basolateral membrane to the apical membrane. In the case of severe ischemia, cell death occurs via necrosis and/or apoptosis. Necrotic debris and detached viable cells are released into the lumen, potentiating renal dysfunction. Viable cells undergo division to replace lost cells, and subsequent differentiation to restore nephron structure and function. Adapted from (41).

Biomarkers of AKI

As discussed above, the earlier an intervention can be instituted for patients with AKI, the more favorable the outcome. As a result, there has been a tremendous investment to identify novel plasma and serum proteins that may characterize the course of renal injury. SCr, as well as blood urea nitrogen (BUN) have been historically used in the clinical setting because both endpoints are easy and inexpensive to measure (49). However, SCr and BUN are not ideal biomarkers as they are both influenced by multiple non-renal factors such as age, nutrition status, muscle mass and metabolism and medications (50). In addition, clinical data revealed that large changes in GFR results in small changes in SCr in the first 24-48 hr following AKI. Thus, delayed intervention and underestimation of the degree of injury has also been attributed to the deficiency of SCr as an AKI marker (51). Given the limitations of SCr and BUN as markers of renal function, novel urinary and serum proteins have rigorously been investigated over the past decade. Given the significant number of biomarkers recently elucidated such as interleukin-18 (IL-18), cystatin C and glutathione S-transferase (Figure 1.7), this review will focus on two noninvasive markers, neutrophil gelatinase-associated lipocalin (NGAL) and kidney injury molecule-1 (KIM-1). Both markers are highly upregulated in renal tubules and present in the urine and blood of animals and patients with AKI (51, 52)

Neutrophil Gelatinase-Associated Lipocalin. NGAL is a 25-kD protein expressed and secreted by immune cells, hepatocytes and renal tubular cells following various insults (53). Experimental data demonstrates that NGAL protein expression is rapidly and persistently increased, upwards of 10-fold, in the renal proximal tubule 24 hr following I/R injury in mice. Interestingly, urinary NGAL was detected in mice 3 hr following renal I/R injury, while SCr levels were only elevated after 24 hr following I/R injury (54). Experimental evidence also revealed excretion of NGAL with no change in SCr levels in

a mouse model of mild renal ischemia, indicating that expression of NGAL is correlative with severity and duration of renal ischemia (54). Clinical data also supports NGAL as a powerful early, sensitive marker of AKI, preceding increase in SCr by 1-3 days in pediatric patients. In addition, significantly higher levels of NGAL were associated with the development of AKI in adult patients following cardiac surgery (51, 55, 56). In summary, NGAL has been indicated as a reliable predictive biomarker in acute and chronic settings of AKI.

Kidney Injury Molecule-1. KIM-1 is a type I transmembrane glycoprotein with an immunoglobulin-line domain and mucin-rich extracellular region conserved across rodents, primates and humans (57). Following ischemic and toxic renal injury, the extracellular domain of KIM-1 is detached and extracted into urine, consequently, a quantitative marker of AKI (51). It is important to note that KIM-1 robust expression is largely restricted to proximal tubule epithelial cells, so, it is also a specific indicator of proximal tubule injury (52, 58). In a mouse model of I/R-induced AKI, both KIM-1 mRNA and protein expression increased with decline of renal function (59). In addition, laboratory evidence revealed that KIM-1 increases apoptosis by facilitating phagocytosis and clearance of apoptotic debris, in turn, dampening the proinflammatory response (60). Rodent studies have also been translated into clinical studies demonstrating 5-fold increase in urinary KIM-1 within 24 hr of renal tubular injury, with no change in serum SCr and BUN until 72 hr following tubular insult (58). Studies have also indicated that KIM-1 excretion is more specific for I/R renal injury compared to other AKI biomarkers (listed in Fig. 1.7), indicating the increased sensitivity of proximal tubules following ischemic renal injury (58). Due to its organ specificity, sensitivity to minor changes in renal function and injury-dependent expression, KIM-1 is considered an ideal maker of AKI and is an approved by the FDA for pre-clinical drug development for AKI.

Biomarkers of AKI	Location	Model
IL-18	Urine	Mice
		Adults
		Children
KIM-1	Urine	Mice, rats,
		Humans
NGAL	Urine	Mice, rats
		Adults
		Children
Tubular enzymuria, eg, γGT, AP,	Urine	Rats
NAG, glutathione S-transferase, lactate dehydrogenase		Humans
Tubular enzymuria (eg, Meprin-1-alpha)	Urine	Rats
Urine sodium/hydrogen exchanger isoform 3 (NHE3)	Urine	Humans
Cysteine rich protein 61 (CYR61)	Urine	Rats, mice
Low-molecular-weight proteins (eg, β2 microglobulin, α-1 microglobulin)	Urine	Humans
Cystatin C	Serum	Humans
Endothelin	Urine	Humans
Hepatocyte growth factor	Urine	Humans
Type I and II receptors for Tumor necrosis factor-α	Serum	Humans
Cytodiagnostic techniques: collecting duct cells and casts	Urine	Humans
IL-6, IL-8	Urine	Humans (renal allografts)
Liver fatty acid-binding protein (L-FABP)	Urine	Children
Biomarker of GFR	Location	Model
Cystatin C	Serum	Humans

Figure 1.6: Biomarkers of AKI. GFR: glomerular filtration rate, IL: interleukin, KIM-1: kidney injury molecule-1, NGAL: neutrophil gelatinase-assocaited liocalin, γ-glutamyl transpeptidase, AP: alkaline phosphatise, NAG: n-acetyl-glucosaminidase. Adapted from (61).

Clinical Management and Treatment of AKI

There is at present, no effective treatment for AKI, therapeutic strategies are largely limited to control of hypertension and hyperglycemia, volume replacement and renal replacement therapy.

As discussed in previous sections, reduced blow flow to the kidney is a major contributor to the onset and progression of AKI. Thus, vasodilators have been under investigation as a potential therapeutic option to improve renal perfusion. Fenoldopam, a selective dopamine receptor 1 agonist, has been effective in increasing renal blood flow to the renal cortex and medullary regions, particularly effective in patients with hypertension (62). Small doses of dopamine also selectively increases the dilation of renal vasculature, enhancing renal perfusion and urine output (63). More studies are need to explore the true therapeutic efficacy of fenoldopam and dopamine; however, compelling clinical evidence supports the notion that vasodilators alone are not sufficient to improve renal function in AKI patients.

Another supportive strategy for AKI patients where hemodynamic changes and volume depletion contributes to the development and progression of AKI, fluid resuscitation with colloid or crystalloid solutions is commonly used to replenish body fluid (64). Control and optimization of fluid replacement is key to health management to avoid fluid overload, which is a mediator of adverse outcomes in AKI patients. In the unfortunate case of fluid overload, patients are typically prescribed diuretics as an adjunctive therapy (65). Fluid replacement serves as method to minimize complications associated with AKI and not the treatment of AKI.

There is strong experimental and clinical data to support the benefits of loop diuretics such as furosemide for the management of AKI. Loop diuretics are proposed renoprotective agents with the rationale being that diuretics decrease cellular transport

of solute and ions, reducing cellular energy demand and hence potentially preserving cell viability (66). In addition, loop diuretics are also associated with reduced tubular cast obstruction, improving urine flow. However, there is some clinical concern that loop diuretics may also be harmful in some scenarios such as in patients with myocardial dysfunction, a common cause of AKI (67). While diuretics have a number of physiological benefits, data from multiple randomized meta-analyses assessing furosemide and mannitol, an osmotic diuretic in the setting of AKI, are inconclusive in determining the true therapeutic efficacy of diuretics (67, 68).

With no lifesaving therapies, renal replacement therapy, commonly known as dialysis, has become routine in the management of critically ill patients and patients that are unresponsive to other interventions (69). As such, dialysis is the only FDA-approved intervention. Meta-analyses data indicates that early initiation of dialysis is associated with better outcomes; however, clinical tests (measurement of SCr and BUN) for the diagnosis and staging of AKI are not ideal for early diagnosis. Thus, there is an incredible need for the identification of therapeutic targets for the effective treatment of AKI. This review will specifically focus on the mitochondrion as a viable therapeutic target in the setting of AKI.

MITOCHONDRIAL BIOLOGY

Mitochondrial Structure

Mitochondria are structurally distinct from other organelles as they are composed of two membranes comprised of phospholipid bilayers: outer mitochondrial membrane (OMM) and inner mitochondrial membrane (IMM). The OMM is selectively permeable containing channel proteins known as porins for transporting molecules across the membrane. The IMM folds inward to form the cristae and is the location of the oxidative phosphorylation machinery, complexes I-IV of the electron transport chain (ETC) and ATP Synthase β (70). In addition to the functional role of the double membranes, they also give rise to the intermembrane space and the mitochondrial matrix. The intermembrane space maintains the protein gradient established by ETC activity and; the mitochondrial matrix contains metabolic enzymes that regulate the citric acid cycle, fatty acid oxidation and protein synthesis (71). In addition, the mitochondrial matrix houses circular and double stranded mitochondrial DNA (mtDNA), which mtDNA encodes for 37 genes necessary for the architecture and function of mitochondria, including 22 transfer RNAs (tRNAs), 2 ribosomal RNAs (rRNAs) and 13 transport chain proteins (72). It is important to note that due to the limited coding capacity of mtDNA, cross talk between nuclear and mitochondrial genomes is essential to the maintenance of mitochondrial function and homeostasis as over 1,500 of the proteins in the mitochondria are nuclear-encoded and subsequently imported into the mitochondria through protein translocation machinery on the OMM and IMM (73).

Mitochondrial Function

Mitochondria play an integral multifaceted role within the cell, with the most prominent function being the generation of ATP by oxidative phosphorylation. In addition to energy

production, mitochondria are also the main sites of calcium sequestration, fatty acid oxidation, reactive oxygen species (ROS) generation and detoxification, heme and steroid synthesis, cholesterol metabolism and apoptotic cell death (74-79). These complex cellular processes are dependent upon functioning mitochondria, highlighting the importance of mitochondria homeostasis in disease and aging populations.

Mitochondrial Homeostasis

Mitochondrial homeostasis, the proper balance of mitochondrial content and metabolism, is a tightly controlled process mediated by the interplay between mitochondrial biogenesis (MB), fusion and fission and mitochondrial clearance (mitophagy) (80, 81). Mitochondria are highly dynamic organelles that are not generated *de novo*, therefore, fusion and fission play critical roles in the preservation of mitochondrial number and morphology necessary to meet cellular energy demands, especially in the face of injury.

Fusion results in long continuous mitochondrial networks through the mixing of healthy mitochondrial components and DNA with those of damaged mitochondria. Fusion is regulated by mitofusin 1 and 2(Mfn1/Mfn2), both located on the OMM and optic atrophy 1 (OPA1) located on the IMM (82). Mfn2 mediates mitochondria-endoplasmic reticulum interactions by aiding in calcium exchange between the two organelles and has been implicated as an essential regulator for proper mitochondrial transport, localization and calcium flux (83). Mfn1 and Mfn2 deficiency in mice results in improper development of the placenta and consequently, midgestational lethality, owing largely to increased mitochondrial fragmentation (84). Additionally, both Mfn2 and OPA1 are associated with neurodegernative diseases, including Charcot-Marie-Tooth (CMT) and autosomal dominant optic atrophy (DOA) (85-87).

Fission refers to the budding of mitochondria into punctate or sphere shaped units through the interaction of OMM proteins cytosolic GTPase dynamin-related protein 1 (Drp1) and mitochondrial fission 1 (Fis1) (82). During mitochondrial fission, Drp1 is translocated to the mitochondria where it interacts with Fis1, leading to the oligomerization of Drp1 and the facilitation of mitochondrial cleavage. A number of post-translational modifications, modulate the functional properties of Drp1, including phosphorylation, SUMOylation, ubiquitination and S-nitrosylation (88), modulates the functional properties of Drp1. Abnormal Drp1 function has been associated with enhanced intracellular ROS, altered calcium flux and apoptosis (89, 90). Like Mfn-null mice, Drp1 knockout mice die early during embryonic development due to improper synapse formation and suppressed neurite growth (91).

Fission also contributes to quality control by enabling the selective removal of damaged mitochondria. Mitophagy is a tightly controlled process by which injured or dysfunctional mitochondria are isolated from the active pool and undergo lysosomal degradation (92). Mitophagy is generally upregulated with aging and injury. There are a number of signaling pathways that facilitate mitophagy, such as those mediated by FUN14 domain-containing protein 1 (FUNDC1), an integral OMM receptor or OMM proteins, BCL2 interacting protein 3/NIX (BNIP3/NIX), which are both induced under hypoxic conditions. (93). It should be also be noted that BNIP3 has proapototic functions (94), highlighting the interconnectivity of two clearance mechanisms to maintain proper mitochondrial function and homeostasis. The most notable mitophagy pathway is mediated by PTEN-induced putative kinase 1 (PINK1) on the OMM and the cytosolic E3 ubiquitin ligase Parkin (81, 93). When PINK1 accumulates on the OMM, the translocation of Parkin to the mitochondria is triggered. PINK-1-mediated phosphorylation of Parkin activates its ubiquitin ligase activity. Parkin then induces the ubiquitination of OMM proteins, such as

Mfn1/2, targeting the mitochondria for degradation by the proteasome or autophagasomes through the recruitment of p62 and LC3-binding domain (95). While abnormal Parkin is most notable for its role in Parkinson's disease (96). Both PINK1 and Parkin1 deficiency have been linked to mitochondrial defects (97, 98). Because of its importance in maintaining proper mitochondrial homeostasis, it is unsurprising that mitophagy has proven to be cytoprotective under cellular stress conditions, such as in the case of I/R injury.

Mitochondrial Biogenesis

As mentioned above MB is broadly defined as the process by which cells generate, new functional mitochondria (99). The purpose of MB is to not only maintain physiological state, but also increase mitochondrial mass and copy number, and thereby increasing ATP, in response to pathological stressors, exercise, caloric restriction and low body temperatures (100). MB relies on the aforementioned regulators of mitochondrial fusion and fission in order to increase the active pool of mitochondria. MB occurs following the transcription and translation of both nuclear-and mitochondrial-derived proteins, which are then incorporated into newly establish mitochondrial networks. (81). Figure 1.7 illustrates the mitochondrial life cycle: biogenesis, fission/fusion and mitophagy.

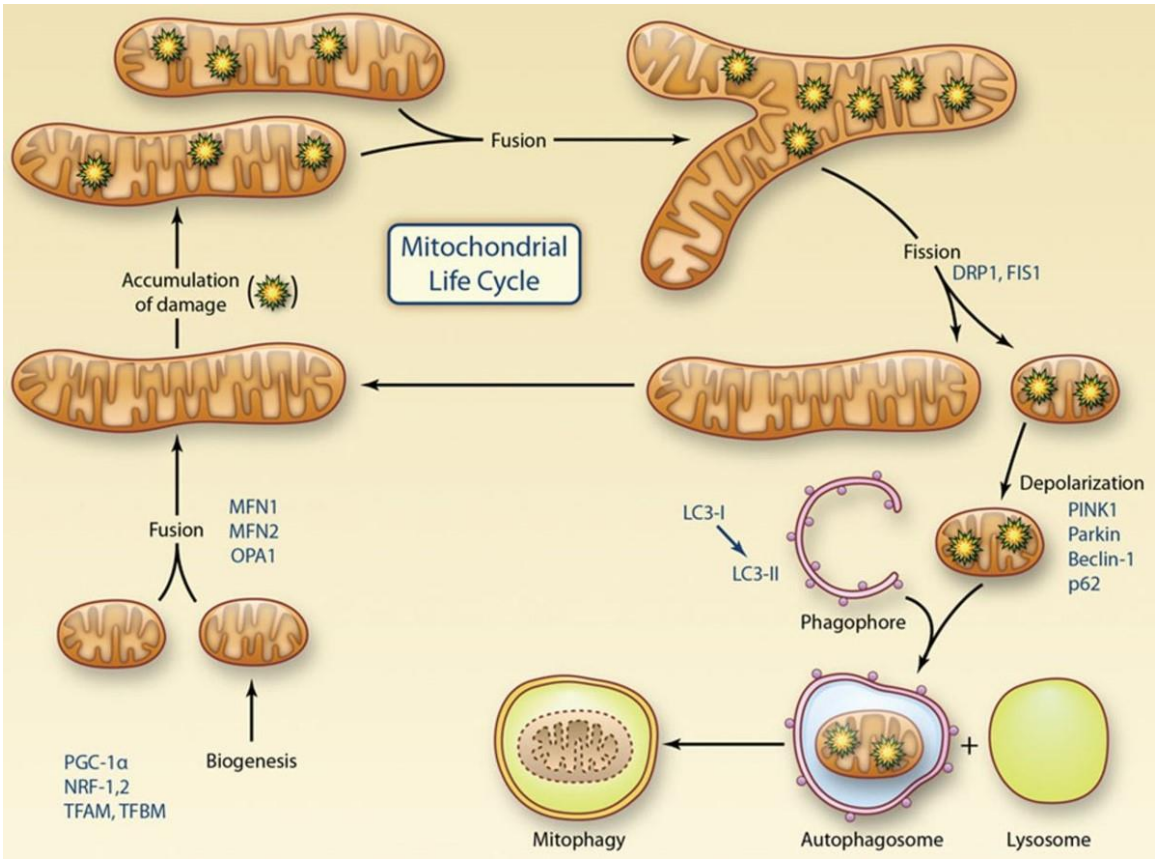

Figure 1.7: Mitochondrial Life Cycle. Mitochondrial homeostasis is a multifactorial process governed by MB, fission/fusion and mitophagy. PGC-1α activates nuclear respiratory factor 1 (Nrf1) and nuclear respiratory factor 2 (Nrf2) to coordinate the expression of nuclear genes required for MB. PGC-1α also activate mitochondrial transcription factor A (TFAM) and mitochondrial transcription factor B (TFBM), which regulate the transcription of genes encoded by mtDNA. Mitochondria undergo cycles of fusion, which is mediated by mitofusin (Mfn) 1, Mfn2 and optic atrophy protein (Opa1). Fission is mediated by dynamin-related protein 1 (Drp1) and fission 1 (Fis1). Fission also promotes the isolation of damaged mitochondria for selective mitochondrial degradation, a process called mitophagy. Mitophagy involves the mitochondrial depolarization, accumulation of putative kinase protein kinase 1 (Pink1) on the mitochondrial membrane, recruitment of Parkin, which targets the mitochondria to the autophagasome through the ubiquitination of mitochondrial proteins. Assembly of the phagasome involves beclin-1, p62 and conjugation of microtubule-associated protein 1 light chain 3 (LC3II). Adapted from (101).

Transcriptional Regulation of MB

The transcriptional regulation of MB is largely governed by the peroxisomal proliferator-activated receptor γ coactivator 1 (PGC-1) family of transcriptional co-activators, including PGC-1α, PGC-1β and PGC-1 related coactivator (PRC), which are all selective regulators of MB and respiratory function (102-104). PGC-1 members are characterized by an N-terminal activation domain with a leucine-rich LXXLL motif that medicates their interaction with various nuclear transcription factors (105). The PGC-1 co-activators do not directly bind DNA, but instead serve as docking platforms that recruit additional protein complexes responsible for the coordination of both the nuclear and mitochondrial genomes (104).

PGC-1α is termed the “master regulator” of MB and is highly expressed in tissues with highmetabolic demand including the skeletal muscle, heart, brown adipose tissue (BAT), brain and kidney (102, 106-108). Activation of PGC-1α not only activates the expression of genes involved in MB, but also activates the expression of antioxidant enzymes such as catalase, superoxide dismutase (MnSOD) and glutathione peroxidase, indicative of the extensive role of PGC-1α extends in the regulation of mitochondrial health (109). PGC-1α was initially described as a coactivator for peroxisomal proliferator-activated receptor γ (PPARγ) in a yeast two-hybrid screen and subsequently as a PPARγ-interacting protein in brown adipose tissue (BAT) to regulate adaptive thermogenesis (108). PGC-1α has recently emerged as a co-activator of several nuclear transcription factors including PPARs (PPARα, PPARδ, PPARγ), nuclear respiratory factors (NRF-1 and NRF-2), forkhead box protein O3 (FOXO3a), as well as nuclear hormone receptors estrogen related receptors (ERRα, ERRβ and ERRγ) (110, 111).

Nuclear Respiratory Factors (NRF-1 and NRF-2). NRF-1 was originally discovered as a transcriptional regulator of cytochrome c and has since been associated with the

expression of a variety of genes required for subunits of respiratory complexes I-V (112). Similarly, gene expression of machinery involved in heme biosynthesis, protein import and assembly (TOM20, TOMO70 and COX17), mitochondrial translation (ribosomal proteins, tRNA synthetases) and mitochondrial transcription (mitochondrial transcription factor A, TFAM) are also NRF-1 dependent. NRF-2 was initially characterized by its direct interaction with the cytochrome c oxidase subunit IV (COXIV) promoter (113, 114). Recent experimental evidence revealed that NRF-2 also exerts its effects through modulating the expression of genes involved in oxidative phosphorylation, mitochondrial transcription factors (TFAM, mitochondrial transcription factor B isoforms) and receptor complexes involved in protein import (TOM20 and TOM70) (113).Homozygous knockouts of NRF-1 are embryonic-lethal. Specifically, NRF-1 null blastocysts exhibit mtDNA depletion and disrupted mitochondrial membrane potential, highlighting the importance of NRFs in the development of proper mitochondrial function and biogenesis (115). Despite the overlapping functions of NRF-1 and NRF-2, experimental evidence demonstrates that NRF-2 cannot rescue the embryonic-lethal phenotype in NRF-1 knockout mice, indicating that NRF-1 and NRF-2 have differential expression during developmental stages and modulate specific transcriptional programs (116). In summary, overwhelming evidence indicates that NRFs play a vital role in mitochondrial function and homeostasis.

Peroxoisome Proliferator-Activated Receptors (PPARα, PPARγ and PPARδ). PPARs are nuclear ligand-activated transcription factors that are key in modulating expression of genes involved in lipid and fatty acid metabolism. The PPAR family is comprised of three isoforms PPARα, PPARγ and PPARδ which have distinct tissue distributions and functions. PPARα is primarily expressed in the liver, kidney and heart and promotes the utilization and catabolism of fatty acids by upregulating genes involved in fatty acid

transport and mitochondrial fatty acid β-oxidation (117). PPARδ is expressed mainly in the brain, adipose tissue and skin where it also promotes uptake and oxidation of fatty acids (118, 119). Because β-oxidation produces substrates for the citric acid cycle and ETC, PPARα and PPARδ are also implicated regulating energy homeostasis and mitochondrial function. PPARγ is highly expressed in white and brown adipose tissue and endothelial cells, with a function of adipocyte proliferation and differentiation, as well as fatty acid synthesis and lipid transport. All PPARs homodimerize with retinoid X-receptor-α and subsequently binds to the promoter regions of target genes such as uncoupling protein 1 (UCP1) and UCP2 (120, 121). UCPs are IMM transporters responsible for dissipating the electrochemical gradient generated by the respiratory chain, and therefore, play a major role in oxidative phosphorylation and adaptive thermogenesis. In terms of promoting MB, PPARs, specifically PPARδ and PPARγ, serve as powerful inducers of PGC-1α gene transcription, acting through a PPAR-responsive element (PPRE) in the distal promoter and successfully induce MB in adipose tissue and skeletal muscle (122). In summary, PPARs are essential in an array of mitochondrial functions from lipid oxidation to MB, demonstrating their importance in mitochondrial homeostasis.

Estrogen-Related Receptors (ERRα, ERRβ and ERRγ). Estrogen-related receptors (ERRs) are orphan nuclear receptors that target a vast number of genes involved fat and glucose metabolism, as well as and MB and function (123). ERRα is the most prominent ERR isoform and is abundantly expressed in highly oxidative tissues. ERRα promotes β-oxidation, citric acid cycle progression, oxidative phosphorylation and ATP synthesis through its control of the medium chain acyl-coenzyme A dehydrogenase (MCAD), fumarate, cytochrome c and subunits of ATP synthase, respectively (124). Through its involvement in fatty acid oxidation and by serving as a binding partner to PGC-1α, ERRα

regulates both oxidative phosphorylation and MB. In fact, experimental inhibition of ERRα disrupts PGC-1α-mediated transcriptional programs, indicating that ERRα is necessary for the induction of MB (125). It is noteworthy to mention that there are cell type-specific differences in the manner by which ERRα regulates mitochondrial function and biogenesis. For example, ERRα may operate in concert with other transcriptional coactivators (NRF2 and PPARα) or may operate independently (126). To further substantiate the importance of ERRs in orchestrating MB and function, ERR-null mice exhibit reduced expression of genes involved in oxidative phosphorylation and altered ETC complex activity in a variety of tissue types including the heart, skeletal muscle and adipose tissue (127-129). These experimental findings support the notion that ERRs are key transcriptional modulators in regulating mitochondrial oxidative phosphorylation and biogenesis.

Mitochondrial Transcription and Replication in MB

As mentioned above, mitochondria are unique in that they contain their own genome encoding for 13 components of the ETC and RNA components required for mitochondrial translation. PGC-1α helps orchestrate mitochondrial transcription and replication by modulating the expression of key players from the nuclear and mitochondrial genomes, such as the mitochondrial DNA polymerase and helicase. This section will briefly discuss the machinery responsible for the transcription and replication of mtDNA for MB.

mtDNA Transcription. The mitochondrial genome is transcribed by RNA polymerase (POLRMT), TFAM and one of two mitochondrial transcription factor B isoforms (TFB1M). Transcription of mtDNA is initiated when POLRMT binds to either of three promoters, heavy strand (HSP) 1 and 2 or light strand (LSP), followed by TFAM recruitment. It has been reported that TFAM may structurally alter and relax by relaxing the mtDNA

exposing the promoter region to facilitate binding of POLRMT and other transcription complexes such as TFBM-POLRMT (130, 131). POLRMT activity yields full-length mtDNA that are subsequently cleaved into mRNAs, tRNAs and rRNAs.

mtDNA Replication. mtDNA replication, DNA helicase (Twinkle), DNA polymerase γ (POLγ), single-stranded binding protein (mtSSB), RNAse H1, DNA ligase III and mitochondrial topoisomerase 1 (TOP1MT), the machinery necessary for mtDNA replication, are all nuclear-encoded and responsible for unwinding, DNA synthesis and repair of mtDNA. mtDNA replication is also intimately linked to transcription through POLRMT and TFAM synthesis of RNA primers used by POLγ for the initiation of replication. It is noteworthy to mention that POLγ is the only mtDNA polymerase and its catalytic domain contains DNA polymerase activity, but also exonuclease and 5'dRP lyase activity, promoting its participation in mtDNA damage censoring and repair (132).

Physiological Regulation of PGC-1α in MB

An extensive amount of attention has focused on understanding the molecular mechanisms responsible for the regulation of PGC-1α expression and activity. A vast range of physiological cues and signals have been elucidated, however, these pathways are both tissue-and environmental-dependent. Major signaling events, including post-translational modifications of PGC-1α are discussed below.

*cAMP-CREB .*A large body of evidence has emerged characterizing the cyclic AMP (cAMP) response element-binding protein (CREB). CREB is a well-studied positive regulator of PGC-1α, which act on the conserved CRE in the PGC-1α promoter. In hepatocytes, PGC-1α expression is induced downstream of glucagon signaling, which leads to increased cAMP, protein kinase A (PKA) and CREB in response to dietary alterations (133). Cold temperatures and exercise activates the β2 adrenergic receptor

leading to enhanced cAMP-PKA-CREB signaling and the subsequent in PGC-1α expression (103). In addition, increased cellular calcium induces CREB activation and ultimately PGC-1α expression via calcium/calmodulin-dependent protein kinase IV (CaMKIV) (134, 135).Calcium also mediates PGC-1α expression, independent of CREB, through the interaction of calcineurin A with the myogenic transcription factors myocyte enhancer factor 2C (MEF2C).

p38. Another mechanism regulating PGC-1α expression involves p38 mitogen-activated protein kinase (p38 MAPK), which is activated during muscle contraction and exercise (136, 137). p38 MAPK-dependent activation of PGC-1α can occur through both MEF2 and activating transcription factor 2 (ATF2). ATF2 is a transcriptional activator of PGC-1α, that when phosphorylated by p38 MAPK, binds to CRE within the PGC-1α promoter. p38 MAPK-dependent activation of ATF2 often occurs in BAT during cold exposure, indicating the role of ATF2 in adaptive thermogenesis (103).

AMPK. AMP-activated protein kinase (AMPK) is a well-studied inducer of PGC-1α and MB. AMPK is a cellular energy sensor and is activated when AMP/ATP ratio is high, ultimately triggering a vast range of pathways to increase cellular levels of ATP. During exercise, ATP levels decrease, in turn, activating AMPK in the muscle (138). A number of reports have revealed that activated AMPK induces MB through the induction of PGC-1α in skeletal muscle. While further studies are needed to elucidate the mechanisms by which AMPK activation increases PGC-1α transcript levels, AMPK has been studied as a pharmacological target for the activation of MB (138).

Nitric Oxide. Activation of nitric oxide synthase (NOS) in BAT and endothelial cells via the generation of cyclic GMP (cGMP) has also been suggested as a potent induce of PGC-1α expression (139). There are three isoforms of NOS (endothelial: eNOS, inducible: iNOS and neuronal: nNOS), and recent evidence has implicated eNOS has a

regulator of MB by increasing PGC-1α, NRF-1 and TFAM mRNA expression, as well as mtDNA content in human cells (139). In particular, eNOS increases cGMP levels through guanylate cyclase, leading to induction of gene transcripts involved in MB though an unknown mechanism.

Receptor-interacting protein 140. Experimental evidence characterizes receptor-interacting protein 140 (RIP140) as a corepressor of PGC-1α by interacting with ERRs and PPARs to antagonize gene expression of PGC-1α and its downstream targets. RIP140-deficient mice exhibit increased mitochondrial gene expression and oxidative capacity; in fact, these transgenic mice are protected against diet-induced obesity due to the increased energy expenditure (140, 141). RIP140 is known to be a regulator of oxidative metabolism in both skeletal muscle and adipocytes (142, 143)

Extracellular signal-regulated kinase. The MEK/ERK signaling cascade has also been implicated as a negative regulator of PGC-1α and MB, particularly under pathological conditions. ERK1/2 reduces PGC-1α and its downstream targets in a number of disease models. In animal models of Parkinson's disease (PD), TFAM phosphorylation as a result of ERK1/2 activation decreased mtDNA transcription (144). In a mouse model of Alzheimer's disease (AD), ERK1/2 activation impaired mitochondrial function, as evidenced by altered mitochondrial fission and fusion and reduced mitochondrial membrane potential (145). Of particular interest, following administration of the oxidant tert-butyl hydroperoxide (TBHP) to RPTC, ERK1/2 phosphorylation correlated with reduced complex I activity and ATP production (146). Furthermore, ERK1/2 activation preceded the decrease in renal PGC-1α mRNA and loss of renal function in a mouse model of I/R-induced AKI (147). Pharmacological inhibition of ERK1/2 phosphorylation has been implicated as protective in a number of animal disease models, indicating the importance of ERK1/2 signaling in mitochondrial homeostasis and cell survival.

Post-translational Modifications of PGC-1α. PGC-1α is also regulated through a host of post-translational modifications, including methylation, acetylation, small ubiquitin-like modifier (SUMO)-ylation, phosphorylation and ubiquitination. Deacetylation by silent mating type information regulation 2 homologue (SIRT1) and methylation by protein arginine methyltransferase 1 (PRMT1) increases PGC-1α activity, whereas acetylation by general control nonderepressible 5 (GCN5) and SUMOylation by SUMO1 negatively impacts the function of PGC-1α (148-151). Phosphorylation of PGC-1α can either increase or decrease its activity depending on the phosphorylating kinase. For example, p38 MAPK and AMPK phosphorylation increases PGC-1α activity, while AKT and GSK3β phosphorylation inhibits its activity (152, 153). Ubiquitination of PGC-1α via an E3 ubiquitin ligase, Skp1/Cullin/F-box-cell division control 4 (SCF^{Cdc4}), targets PGC-1α for proteasomal degradation (154). These modifications allow for the fine-tuning of PGC-1α activity in a context-dependent manner.

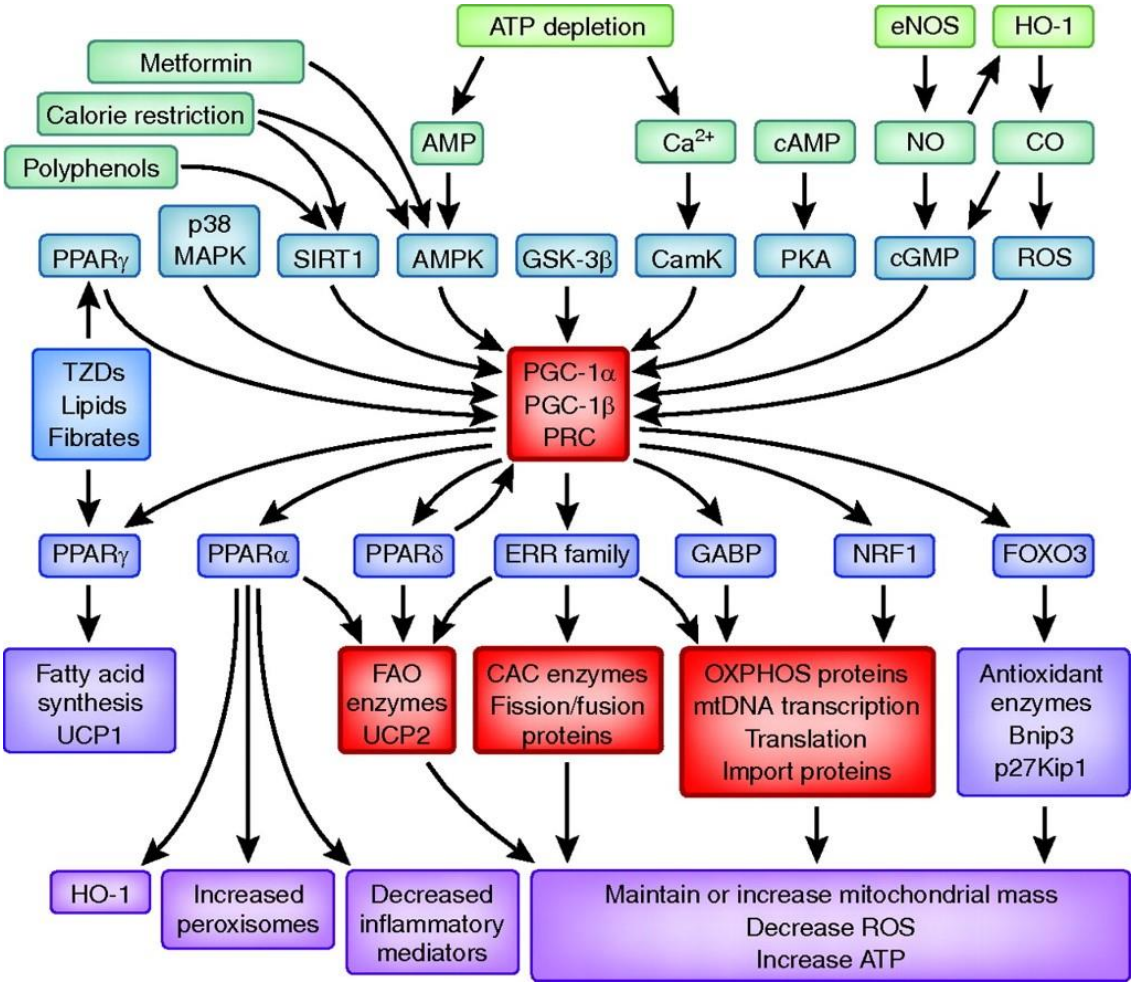

Figure 1.8: Regulation of PGC-1α and MB. A summary of the pathways involved in the promotion and mediation of MB through modulating PGC-1 transcription, post-transcriptional status, or both as described above. Because of the complexity of the pathways involved, many intermediates and feedback interactions are not included. Inhibitory factors, such as receptor-interacting protein 140 (RIP140), general control nonderepressible 5 (GCN5) and Akt are not shown. Red boxes indicate essential targets required for MB. AMPKP: AMP-activated protein kinase, CAC: citric acid cycle, CamK: calcium-calmodulin-dependent protein kinase, CO: carbon monozide, eNOS: endothelial nitric oxide synthase, FAO: fatty acid oxidation, GABP: GA-binding protein α, GSK-3β: glycogen synthase kinase-3β, HO-1: heme oygenase 1, NO: nitric oxide, OXPHOS: oxidative phosphorylation, PKA: protein kinase A, PRC: PGC-1 related coactivator, TZDs: thiazolinediones, UCP: uncoupling protein. Adapted from (111)

MITOCHONDRIAL DYSFUNTION AND BIOGENESIS IN AKI

Renal Proximal Tubule Bioenergetics

Active transport of solutes in proximal tubules require high turnover of ATP derived almost exclusively through mitochondrial oxidative phosphorylation. In fact, at least 70% of mitochondrial oxygen consumption is utilized by the renal proximal tubule to drive the ATP-dependent sodium pump and establish the chemical gradients necessary for reabsorption (155). The proximal tubule primarily oxidizes fatty acids, amino acids and pyruvate to fuel oxidative phosphorylation to meet this constant metabolic demand (155). It is important to emphasize that the proximal tubule does not rely on glycolytic metabolism, thus, the major regulatory enzymes of glycolysis have very low activity in the renal cortex (156). As the proximal tubule is the most metabolically active epithelia in the kidney, it is unsurprising that AKI, whether nephrotoxic, septic or ischemic in origin, involves pathological changes in the mitochondria, in turn, contributing extensively to renal dysfunction and damage (157, 158)

Evidence of Mitochondrial Dysfunction in I/R-induced AKI

Mitochondria have been a major focus for understanding the pathophysiology of I/R-induced AKI. There is overwhelming evidence indicating mitochondrial dysfunction as an initiator of renal dysfunction and damage in both cellular and animal models. I/R-induced mitochondrial alterations include reduction in ATP-generating capacity, alterations in mitochondrial dynamics, enhanced ROS production and apoptosis.

Decline in ATP Content. AKI is commonly characterized as a state of tubular ATP depletion, which has been linked to impaired oxidative phosphorylation and loss of mitochondrial membrane potential (158-162). Mice subjected to I/R-induced AKI exhibit protein reduction in oxidative phosphorylation subunits such as cytochrome c oxidase I (COXI), NADH dehydrogenase [ubiquinone] 1 beta subcomplex 8 (NDUFB8) and ATP

Synthase β, subunits of complexed I, IV and V, respectively. In addition, alterations in complex I activity and loss of mitochondrial membrane potential was observed in isolated proximal tubular cells following hypoxia-reoxygeneation (160, 163). It should be noted that reduction in energy production following I/R-induced AKI prevents not only the transportation of solutes, but also energy-dependent repair processes and initiates additional pathological events, including loss of renal brush border, calcium accumulation, apoptosis and mitochondrial fragmentation (159, 164-166). Taken together these findings highlight the important role of mitochondrial dysfunction in the pathophysiology of is I/R-induced AKI.

Reactive Oxygen Species Generation. ROS formation in the kidney plays a significant role in ischemia/perfusion, cisplatin, mercury and glycerol models of AKI (167-171). Electron leakage in the mitochondrial respiratory chain and activation of leukocytes are largely responsible for the generation of ROS following ischemia (172, 173). While mitochondria are a major source of ROS generation, mitochondria are also a primary target for ROS-mediated damage. ROS reacts with proteins, lipids and nucleic acids contributing to sublethal cellular injury and apoptosis (174). In particular, mtDNA is extremely susceptible to oxidative damage due to its proximity to ROS generated by complex I and III of the electron transport chain, mitochondria-localized dehydrogenases, oxidases and enzymes involved in β-oxidation (78, 175). Renal I/R injury also impairs ROS scavenging processes as demonstrated mitochondrial superoxide dismutase 2 (SOD2) reduction. In turn, overexpression and/or induction of SOD2 has resulted in renoprotective effects through attenuation of oxidative stress and renal dysfunction in animal models of I/R-induced AKI (176). Additionally, positively charged antioxidant agents that selectively accumulate in the mitochondrial matrix, such

as SkQ1 and SkQR1 provided renoprotection under I/R conditions, indicating the significant impact of ROS on tubular function in the setting of AKI (177).

Apoptosis. Mitochondria are also key regulators of apoptotic signaling in response to renal ischemic and nephrotoxic insults. Mitochondria regulate caspase activation and cell death through a process called mitochondrial outer membrane permeabilization (MOMP) (178). MOMP is highly regulated by the pro-apoptotic Bcl-2 family members, such as Bax, which inserts into the outer membrane of the mitochondria, increasing mitochondrial permeability to proteins, allowing for cytochrome c release (179). Cytochrome c then interacts with the caspase adaptor molecule apaf-1, in turn, recruiting pro-caspase-9 and the subsequent activation of caspase 9 (179). Activated caspase 9 is responsible for the cleavage and activation of the executioner caspases-3 and -7 and the subsequent proteolytic cleavage of cellular components, ultimately leading to cell death (179, 180). It is noteworthy to mention that the two additional pro-apoptotic members of the Bcl-2 family, Bid and Bad, also translocate to the mitochondria in response to I/R renal injury and subsequently activates proteolytic caspases similar to Bax (181). Plotnikov et al demonstrated that the translocation of Bax to the mitochondria increased by 50% after renal I/R in rats and this was directly associated with a 40% reduction in cytochrome c content in the mitochondria (182). Treatment with diosmetin, an anti-inflammatory and anti-apoptotic flavonoid, protected against tubular damage, renal dysfunction and cell death following I/R-induced AKI (183). These studies, among several others, provide strong evidence that enhanced apoptotic cell death is a major determinant in the pathophysiology of AKI.

Altered Mitochondrial Dynamics. Mitochondrial size and integrity are important aspects when considering the role of mitochondrial in the setting of AKI. As mentioned earlier, mitochondrial are dynamic organelles that are continuously fusing and dividing in

response to cellular needs. An increasing level of attention has been given to mitochondrial fragmentation, or fission, and how it contributes to mitochondrial damage and subsequent cell death. Under stress conditions, such as I/R injury, Drp1 translocates from the cytosol to the OMM, where it oligomerizes to drive OMM constriction and cleavage (184, 185). The activation and recruitment of Drp1 to the mitochondria is mediated by a number of post-translational modifications as well as its interaction with Fis1 (186). It is also important to discuss the functional link between loss of mitochondrial membrane potential, mitochondrial fission and apoptosis. A number of studies have documented changes in mitochondrial morphology as a result of ATP depletion and MOMP (187, 188). Addtionally, mitochondrial fragmentation is thought to occur during the cell death pathway (188). Experimental inhibition of Drp1 prior to the initiation of apoptosis not only inhibited mitochondrial fission but also delayed caspase activation and cell death (188, 189). In particular, Brooks et al reported enhanced mitochondrial fission in renal tubules in response to I/R injury(158). Additional studies revealed the administration of Mdivi-1, a pharmacological inhibitor of Drp-1, reduced mitochondrial fragmentation, having conferred beneficial effects in experimental models of AKI by blocking cell death (158). In summary, the role of mitochondrial is multifactorial and multiphasic in the setting of AKI; therefor, minimizing these deleterious mitochondrial events has the potential to reduce injury and improve cellular energetics, integrity and function.

Altered MB in I/R-induced AKI

Intensive exploration has focused on understanding the underlying mechanism leading to mitochondrial dysfunction following AKI. In particular, our laboratory has focused on the disruption of MB as a key mediator of mitochondrial dysfunction. As discussed earlier, MB, through the actions of PGC-1α, is responsible for regulating the

transcriptional and translational programs required for the proper maintenance of mitochondrial content and function under physiological and pathological conditions. The study of PGC-1α and MB is particularly important when assessing tubular mitochondrial homoeostasis as renal PGC-1α is primarily localized in the proximal tubule in order to meet the high cellular energy demand (190). We demonstrated early reduction in PGC-1α and PGC-1β mRNA expression followed by sustained depletion of respiratory chain proteins NDUFB8, ATP Synthase β and COX1 in the renal cortex, which is mostly comprised of proximal tubules, of mice following I/R-induced AKI (191)(Fig. 1.9). In addition, these animals also exhibited increased Drp1 protein expression and procaspase 3 cleavage, indicating increased mitochondrial fragmentation and apoptosis (191). In a subsequent study, depletion of renal cortical mtDNA content was also noted in a mouse model of I/R-induced AKI (192). It is important to note that suppression of PGC-1α and MB has also been witnessed in other models of AKI. For instance, reduction of PGC-1α expression and its downstream targets was observed in a mouse folic-acid model of AKI and this was correlated with persistent suppression of renal cortical mtDNA copy number (193). In addition, Smith et al. demonstrated reduced renal cortical PGC-1α mRNA and protein levels, along with decreased respiratory chain protein expression, correlated with tubular injury as evidenced by KIM-1 and renal dysfunction as evidenced by BUN in a endotoxin model of AKI (194). Taken together, these findings indicate that PGC-1α and MB serve as potential targets to rescue mitochondrial content and function in the setting of AKI.

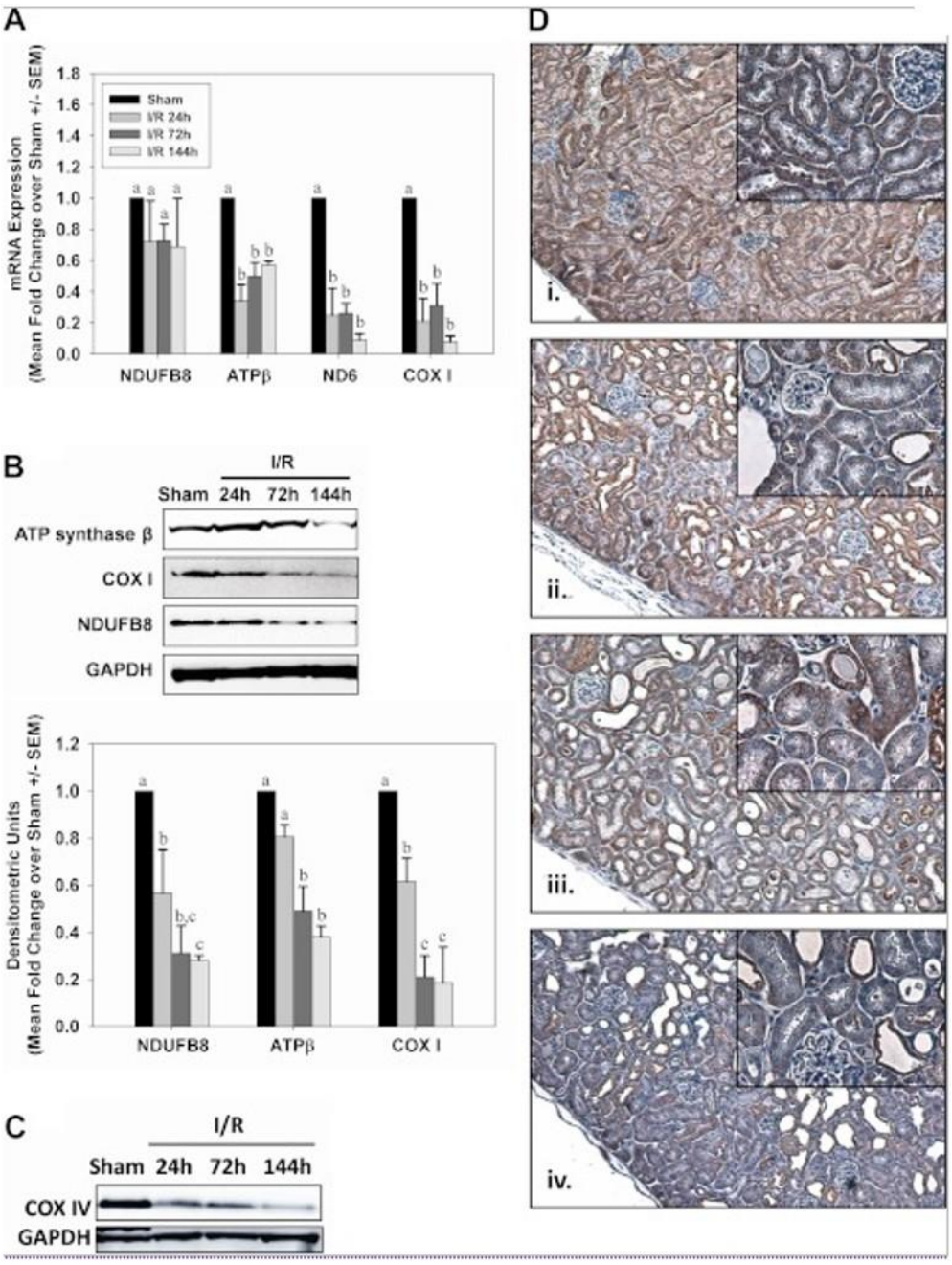

Figure 1.9: Disrupted MB in I/R-induced AKI. Renal cortical gene expression of nuclear-encoded respiratory components, NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 8 (NDUFB8) and ATP Synthase β (ATPβ) were assessed in sham and I/R- injured mice at 24, 72 and 144 hr following injury (A). Protein expression of renal cortical NDUFB8, ATPβand COX1 was also assessed in the same animals (B). Sustained depletion of genes and proteins required for respiratory chain function was observed in mice subjected to renal I/R injury. Immunoblot and immunohistochemical analysis (C, D) of cytochrome c oxidase IV (COXIV) was also assessed, following the same pattern as other respiratory proteins.

Pharmacological Induction of MB

The most convincing data that illustrates the true potential of MB as a viable target following AKI is depicted in studies utilizing pharmacological induction of MB and subsequent restoration in mitochondrial homeostasis and renal function. A proof-of-principle experiment was perform by our laboratory in rabbit primary RPTC that were exposed to H_2O_2 followed by the overexpression of PGC-1α (195). PGC-1α overexpression restored respiratory chain protein expression, cellular respiration and ATP content, as well as facilitated the recovery of sodium transport in oxidant treated RPTC (195). Collectively, this study provided evidence that the induction of MB is capable of reversing mitochondrial dysfunction, which may be driving the recovery of normal proximal tubule cellular function following acute oxidant injury.

Our laboratory previously demonstrated that isoflavones, cGMP-specific phosphodiesterase (PDE) inhibitor and 5-HT_2 pan agonist 1-(2,5-dimethyoxy-4-iodophenyl)-2-aminopropane (DOI) promoted the increase of nuclear-encoded and mitochondrial-encoded proteins involved in MB in RPTC *in vitro* (196, 197). Additionally, pharmacological activators of SIRT1 deacetylated PGC-1α, thereby, increasing the active pool of PGC-1α, and attenuating renal and mitochondrial dysfunction in a rat model of I/R-induced AKI (198). Lastly, treatment with formoterol and sildenafil (phosphodiesterase, PDE5 inhibitor) beginning 24 hr following bilateral renal I/R injury or folic acid-induced AKI, respectively, accelerated mitochondrial and renal function recovery in mice (193, 199). Taken together, these studies strongly support the notion that the induction of MB serves as a viable treatment for the restoration of mitochondrial homeostasis for the consequent recovery of tubular and renal function following AKI. Despite the overwhelming amount of preclinical evidence, relatively few drugs have been identified for the induction of MB. Thus, there is a tremendous need for the

identification of novel therapeutic targets that not only stimulates MB but also extend beyond partial restoration of cellular function. Figure 1.10 summarized pharmacological approaches for the stimulation of MB.

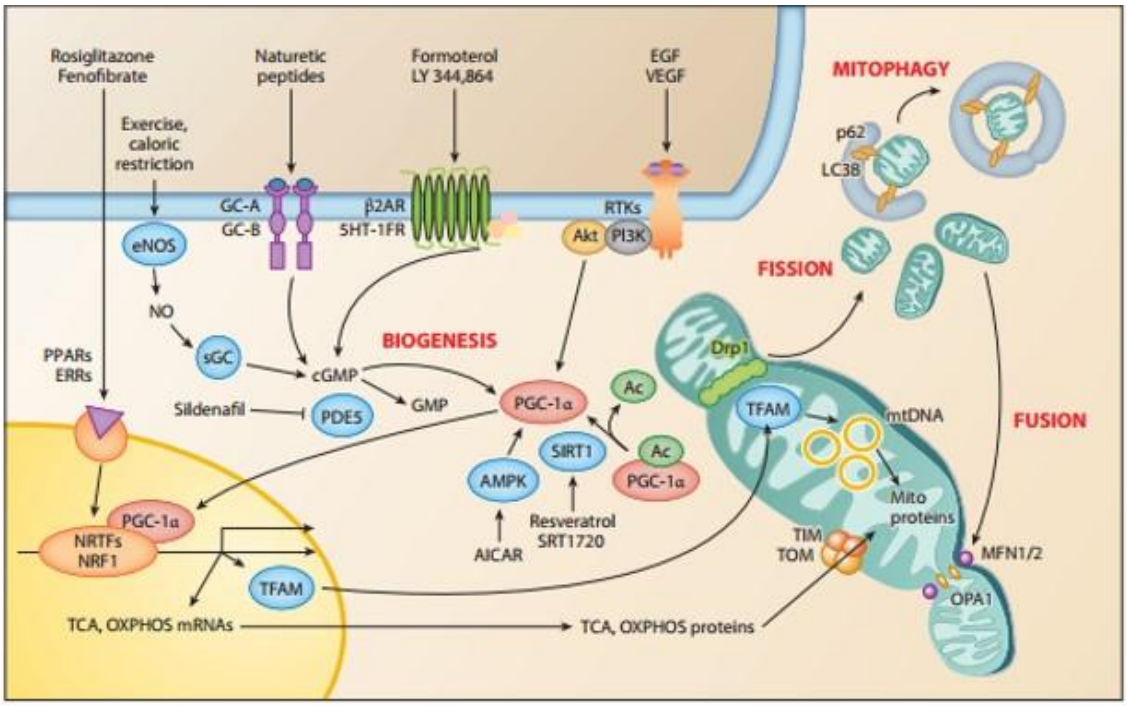

Figure 1.10: Pharmacological Activation of MB. MB is a complex process controlled by the transcriptional regulator peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC-1α). PGC-1α expression and activity is regulated by a variety of pathways including receptor tyrosine kinases, G-protein coupled receptors (GPCR), naturetic peptide receptors, cyclic guanosine monophosphate (cGMP) , as well as sirtuin (SIRT)1-mediated deacetylation. In response to various stimuli, mitochondrial undergo the process of mitochondrial fusion via mtofusin (Mfn) 1, Mfn 2, optic atrophy 1 (Opa1) or fission via a host of proteins such as dynamin-related protein 1 (Drp1).Damaged mitochondria are cleared by the selective degradation in lysosomes. EGF: epidermal growth factor, VEGF: vascular endothelial growth factor, RTKs: receptor tyrosine kinases, β2AR: β2-adrenergic receptor, 5HTR: serotonin receptor, GC-A/B: guanylyl cyclase A/B, eNOS: endothelial nitric oxide synthase, NO: nitric oxide, sGC: soluble guanylate cyclase, cAMP: cyclic adenosine monophosphate, AMPK: AMP-activated kinase, PPAR: peroxisome proliferator activated receptor, ERR: estrogen related receptor, NRF1: nuclear respiratory factor 1, Ac: acetyl, TCA: tricarboxylic acid cycle, OXPHOS: oxidative phosphorylation, TOM: translocase of the outer membrane, TIM: translocase of the inner membrane, mtDNA: mitochondrial DNA. Adapted from (200).

SEROTONIN AND SEROTONIN RECEPTORS

Serotonin and its Function

5-hydroxytryptamine (serotonin, 5-HT) is a monoamine neurotransmitter synthesized from its precursor amino acid L-tryptophan through a biochemical pathway comprised of three enzymes: tryptophan hydroxylase (TPH), aromatic acid decarboxylase (DDC) and pyridoxal phosphate (201). The metabolism of 5-HT is mediated by the OMM enzyme monoamine oxidase (MAO) and aldehyde dehydrogenase and the metabolites are then excreted by the kidney (202).

While serotonin is best known for its role in the central nervous system (CNS), the majority of 5-HT is primarily found in the periphery with approximately 90% of total body 5-HT released into the gastrointestinal tract or stored in blood platelets (203). Serotonin mediates a vast array of physiological processes including behavior and mood, pain control, hemostasis and platelet function, cardiovascular function and valvular closure, breathing and respiratory drive and gastrointestinal motility.

Central Nervous System. Serotonin modulates a number of behavioral processes though specific serotonin receptors (discussed below). Mainly, serotonin regulates anxiety-like behavior, reward processing, locomotion, appetite and energy balance (204-206). 5-HT also mediates the release of other neurotransmitters such as glutamate and dopamine. In addition, pharmacological inhibition of serotonin reuptake has proven to be viable approach in the management of depression by increasing serotonin pools in the brain (207-209).

Another important role of serotonin in both the CNS and peripheral nervous system (PNS) is its modulation in pain perception and nociceptive processing (210). 5-HT is released within inflamed tissue to mediate the signal transduction of nociceptive

information to the brainstem, cortical and limbic regions, in turn, modulating the psychological perception of pain (210). The 5-HT receptor have been successfully drugged for the treatment of pain, specifically, triptans which were developed for the treatment of migraine symptoms.

Blood Platelets. Blood platelets have a significant storage of serotonin as they lack the machinery to synthesize serotonin *de novo*. Serotonin is taken up from the plasma by blood platelets to promote platelet aggregation and vasoconstriction in the surrounding tissues to facilitate hemostasis. Interestingly, serotonin also has the capacity to activate vasodilation, which is commonly observed following the stimulation of a specific class of 5-HT receptors (discussed below) (211).

Cardiac Tissue. Several studies have documented the role of serotonin in regulating cardiac function. Serotonin modulates the release and activity of several vasoconstrictors, such as angiotensin and norepinephrine, which are critical regulators of systemic and primary pulmonary hypertension (212, 213) . Thus, alterations in the serotonergic system can play a pathological role in multiple cardiovascular diseases. In addition, serotonin's role in cardiac valvular function was first noted after patients prescribed fenfluramine, an appetite suppressant that acts by increasing serotonin, developed valvulopathy and fibrosis (213).

Respiratory System. Early work by Reid and Rand described the role of 5-HT in breathing control mediated by the serotonergic system in the brainstem and pre and postsynaptic 5-HT receptors (214). In a feline model, they observed apnea following intravenous injections of 5-HT (214). Subsequent studies revealed serotonin as a neuromodulator of breathing, facilitator of respiratory rhythm and regulator of CO_2/pH homeostasis (215, 216). Additionally, adverse changes in serotonin levels and signaling have been correlated with severe breathing disorders.

Gastrointestinal Tract. Perhaps the most prominent role of serotonin in the periphery is its role in regulating digestion. In fact, 5-HT is involved in the digestion of food on many levels, from stimulation of taste-bud cells to the induction of intestinal motility and to the secretion of digestive enzymes (pancreatic enzymes) (203, 217, 218). Considering serotonin's involved role in the gastrointestinal activities, it is no surprise that abnormalities in serotonin signaling has been implicated in bowel disorders, such as irritable bowel syndrome (219).

In summary, the serotonergic system is complex with a wide range of modulatory activity of biologic responses. The overwhelming involvement of serotonin is governed by a large class of serotonin receptors that are expressed in both the CNS and PNS. Experimental evidence has linked a number of specific serotonin receptors to a given process, allowing for the opportunity to pharmacologically target these receptors in various disease states. The next section will discuss the various serotonin receptor classes.

Serotonin Receptor Structure and Signaling

Serotonin exerts its effects through the interaction with as many as 13 distinct heptahelical, G-protein coupled receptors (GPCR) and ligand-gated ion channels (220).

Serotonergic Ligand-Gated Ion Channels. The 5-HT_3 receptor family are the only serotonin receptors that are cation-selective (Na+ or K+) channels. Upon interaction with 5-HT, rapid neuronal depolarization occurs and the cation channels open in response, creating an excitatory response (221). 5-HT_3 receptors are expressed in both the CNS and PNS, modulating excitatory synaptic transmission and neurotransmitter release (222, 223).

Serotonergic G-Protein Coupled Receptors. There are six families of class A (rhodopsin-like) GPCR that are modulated by serotonin (5-$HT_{1,2,4-7}$). The 5-HT families are further divided into subclasses based on their distribution, function and structure (220). The 5-$HT_{1,5}$ receptors are coupled to $G\alpha_i/G\alpha_o$, the 5-$HT_{4,6,7}$ receptors are coupled to $G\alpha_s$ and the 5-HT_2 receptors are coupled to $G\alpha_q/G\alpha_{11}$ (220). Briefly, when serotonin or a 5-HT receptor agonists interacts with any of the GPCR mentioned above, a conformational change of the GPCR occurs (224, 225). The now activated Gα protein exchanges GDP for GTP, in turn, triggering the dissociation of Gα subunit from the Gβγ heterodimer from the receptor. The dissociated Gα and Gβγ heterodimer goes on to interact with a variety of intracellular proteins to induced signal transduction cascades (226). Figure 1.11 is a visual representation of this process.

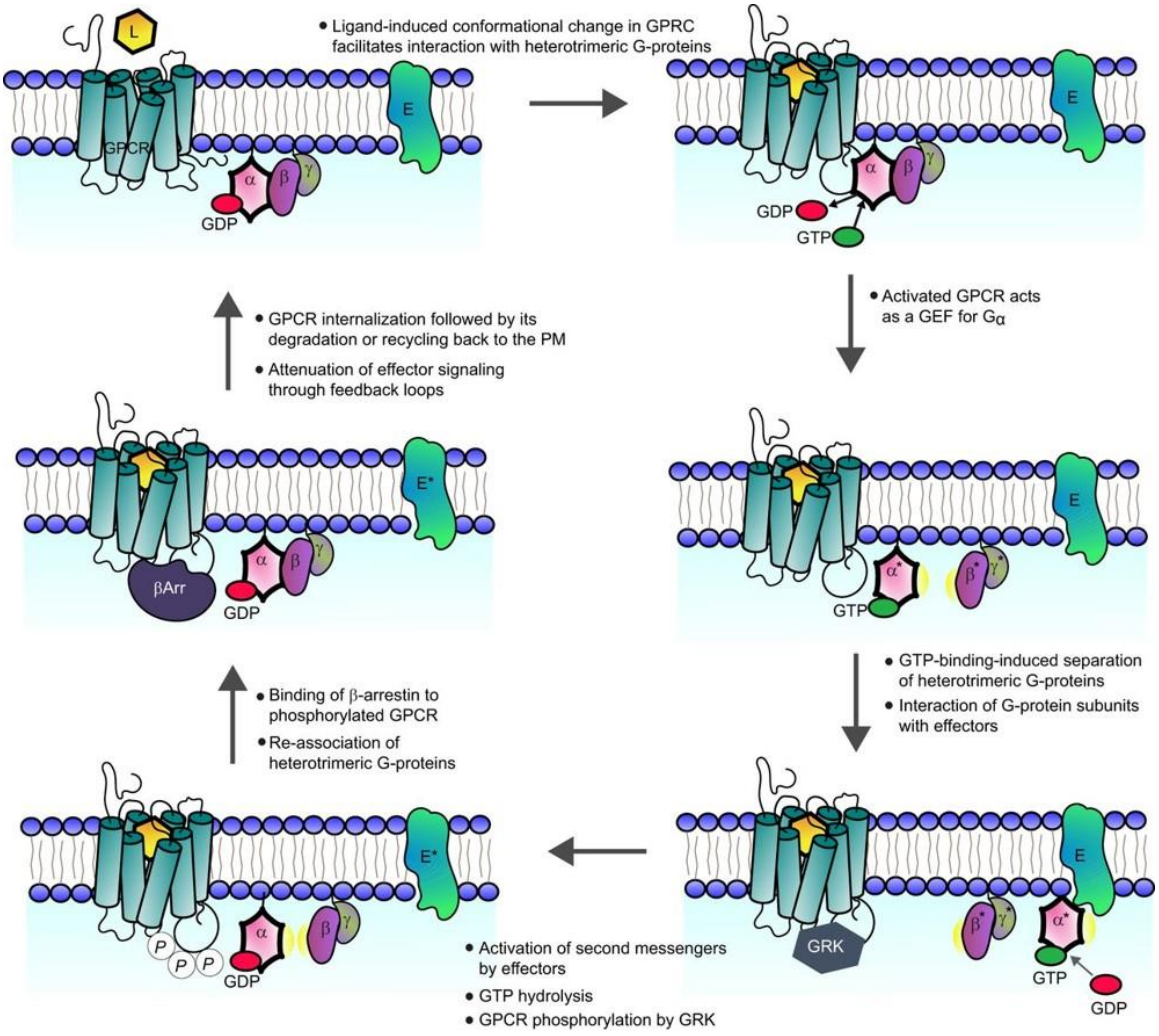

Figure 1.11: GPCR Cycle. Basal conformation of GPCR associated with Gα-βγ heterotrimer (Top Left). Ligand binding activates and induces conformational change of the GPCR allowing exchange of GDP for GTP on the Gα subunit (Top Right). The Gα-GTP and Gβγ subunits dissociate into their active state (Middle Right). Activated subunits can then interact with downstream effectors, for example Gα can interact with an effector (E) (Bottom Right). Gα and Gβγ interaction with an effector leads to the activation of second messengers (Bottom Left). The GTP in Gα is hydrolyzed to GDP, leading to the inactivation of Gα and Gβγ subunits and the reassociation of the Gα-Gβγ heterodimer complex on the GPCR. Once the GPCR is inactive, GRKs and β-arrestin associate with the GPCR to promote internalization of the receptor (Middle Left).GPCR: G-protein coupled receptor, GEF: guanine nucleotide exchange factor (GEF), GDP: guanine diphosphate, GTP: guanine triphosphate, GRK: G protein-coupled receptor kinases, PM: plasma membrane. Adapted from (226).

Intracellular signaling is dependent upon the Gα subunit coupled to the GPCR. Specifically, $G\alpha_{q/11}$ heterodimer activates phospholipase C (PLC) cleavage of phosphoinositol 4,5-biphosphate (PIP2) to diacyl glycerol (DAG) and inositol triphosphate (IP3) for the consequent release of intracellular calcium from the endoplasmic reticulum(220, 227). $G\alpha_s$ and $G\alpha_i$ both modulate cyclic adenosine monophosphate (cAMP) production via activating or inhibiting adenylate cyclase activity, respectively. cAMP is considered a second messenger as one of its primary functions is regulating the activity of the serine/threonine PKA family (208, 220).

It is extremely crucial to mention the signaling role of Gβγ heterodimer upon its dissociation from Gα. While Gβγ heterodimer signaling is vastly understudied, Gβγ heterodimer is known for activating distinct signaling pathways via regulation of ion channels such as N-type calcium channels and direct protein-protein interactions with a variety of different effectors such as PI3K (228, 229). Another important observation is that the activation of Gαi-coupled GPCR releases a great amount of Gβγ to achieve significant effector signaling (228). However, it is important to note that these pathways are both tissue-and context-dependent.

Serotonin in the Kidney

Serotonin is actively synthesized by the proximal tubules in the renal cortex. Renal serotonin has been reported to be involved in renal metabolism and vascular tone and speculated to play a role in inflammatory and fibrotic responses following renal injury (230). Xu et al. characterized the intrarenal serotonergic system, which includes receptors belonging to the $5\text{-}HT_{1,2}$ family as well as the serotonin transporter (SERT), particularly in the proximal tubule epithelial cells (231). This study also revealed

serotonin stimulated the expression of two mediators of extracellular matrix accumulation and vascular endothelial growth factor (VEGF), indicating the possible role of renal 5-HT in fibrosis, however, the full extent of 5-HT signaling in the kidney has yet to be reported (231). The high presence of renal serotonin and its receptors not only suggests a prominent role of serotonin in the kidney but also supports the use of renal 5-HT receptors as therapeutic targets.

Functional Role of Serotonin Receptors in Mitochondria Homeostasis and Biogenesis

The role of serotonin receptors in regulating mitochondrial homeostasis is of particular interest to our laboratory because a phenotypic screening assay revealed agonists of 5-$HT_{1,2}$ receptors induced MB in RPTC. Specifically, DOI enhanced mitochondrial function (cellular respiration, ATP production, mitochondrial membrane potential) and mitochondrial mass (respiratory chain proteins and mtDNA copy number) in RPTC (197). These positive effects on mitochondrial function and content were determined to be dependent on PGC-1α and was blocked when RPTC were treated with a pan-5-HT receptor antagonist (AM-193). Furthermore, DOI restored mitochondrial function (cellular respiration) following oxidant injury in RPTC (197). This work was further extended to reveal that 5-HT_{2C} agonist and antagonist promoted MB in RPTC in a 5-HT_{2A} receptor-dependent manner (232). These studies indicate that targeting serotonin receptors for the induction of MB is a potential therapeutic strategy to promote the recovery of mitochondrial and renal function in the presence of injury.

In addition to our findings, other groups linked the 5-HT receptors to mitochondria. Nebigil et al. extensively characterized the role of the 5-HT_{2B} as a vital regulator of survival signals in cardiomyocytes (233). Interestingly, his worked revealed that the depletion and overexpression of the 5-HT_{2B} receptor altered mitochondrial function and integrity. Specifically, 5-HT_{2B} receptor knockout mice exhibited reduced mitochondrial

enzyme activities (cytochrome oxidase and succinate dehydrogenase), mitochondrial number and swollen cristae. These mitochondrial abnormalities were associated with myocardial cell death (234). Transgenic mice overexpressing the 5-HT_{2B} receptor exhibited hypertrophic cardiomyopathy and dilated cardiomyopathy as a result of increased mitochondrial proliferation and cell size. These mice also displayed increased succinate dehydrogenase and cytochrome c oxidase activities (235). Interestingly, the overexpression of the 5-HT_{2B} receptor yielded a similar phenotype observed in mice that overexpressed cardiac PGC-1α (236).

The 5-HT_3 and 5-HT_4 receptors have also been associated with cardiac mitochondria. Both receptors are functionally located on the mitochondrial membrane and exhibit distinct effects on mitochondrial function (237). Cardiac 5-HT_3 and 5-HT_4 receptors influence mitochondrial calcium uptake, mitochondrial respiration and ATP production as well as modulation of the mitochondrial permeability transition pore. These alterations in mitochondria homeostasis and function were also associated with impaired cardiac behaviors as measured by cardiac rhythm (237). Taken together, these studies suggest that both types of serotonin receptors, ion channel and GPCR, influence mitochondrial function and ultimately essential cellular functions.

Based on the aforementioned findings, our laboratory continued to probe for 5-HT receptor ligands that promote the induction of renal MB with the hopes of identifying a novel target for the recovery of renal function in the setting of AKI.

5-HT_{1F} RECEPTOR

5-HT_{1F} Receptor Structure and Distribution

The 5-HT_{1F} receptor was cloned and sequenced in 1992 and shares the greatest sequence homology with the 5-HT_{1E} receptor (238). The 5-HT_{1F} receptor is a GPCR containing 366 amino acids that inserts into cellular plasma membrane forming four extracellular, seven transmembrane and four cytoplasmic domains (238).

The brain distribution of the 5-HT_{1F} receptor was elucidated using selective 5-HT_{1F} radioligand, [3H]LY344370, revealing 5-HT_{1F} receptor localization in layers IV-V of cortical regions, striatum,olfactory bulb, CA3 hippocampal region, nucleus accumbens and thalamus (239). Peripheral expression of the 5-HT_{1F} receptor has been reported in reproductive, cardiac, hepatic, retinal, renal and intestinal tissues, as well as peripheral blood lymphocytes (238-242). While the 5-HT_{1F} receptor is arguably the most understudied serotonin receptor, its expression has been identified in numerous tissue types, shedding light on the importance of the 5-HT_{1F} receptor in the modulation of physiological and potentially pathological cellular processes.

5-HT_{1F} Receptor Signaling Physiological Roles

The 5-HT_{1F} receptor exerts its cellular effects via forskolin (FSK)-stimulated inhibition of adenylate cyclase activity and the subsequent reduction of cAMP accumulation, a common signaling transduction pathway of 5-HT_1 receptors (243). NIH-3T3 and LM(tk-) cells (cultured mouse fibroblasts) were transfected with 1.7 and 4.4 pmol/mg protein 5-HT_{1F} receptor density, respectively, to explore the functional coupling of the 5-HT_{1F} receptor. Interesting, only 10% receptor occupancy was required to mediate half-maximal response of cAMP inhibition in these cells, illustrating the potent ability of the 5-HT_{1F} receptor in promoting Gαi/o signaling (243). This study also revealed alternative

signal transduction pathways downstream of the 5-HT_{1F} receptor, which appears to be cell or context specific. LM(tk-) cells transfected with 5-HT_{1F} receptor were exposed to relatively small concentrations of serotonin, inducing IP_3 stimulation and the consequent increase in intracellular calcium concentration (243). However, IP_3 activation and calcium release were absent in the NIH-3T3 cells, even at serotonin concentrations as high as 100μM (243). These data suggest that cells possessing high 5-HT_{1F} receptor density have the ability to interact with multiple cellular effectors beyond those of the canonical signaling pathway.

The 5-HT_{1F} receptor has been described as a neuronal receptor of the CNS that mediates pain without vasoaconstrictive effects (244). Experimental studies dedicated to the 5-HT_{1F} receptor has focused on the development of 5-HT_{1F} receptor agonists for the treatment of migraines (244). The trigeminal system is responsible for carrying pain impulses from the face, neck, teeth and ears to the central nervous system, thus, it is a primary target for the treatment of migraines (245). The 5-HT_{1F} receptor is highly expressed in the blood vessels and nerve endings of the trigeminal system, therefore, targeting the 5-HT_{1F} receptor has proven to be an effective strategy in the augmentation of migraine pain (244, 246, 247).

5-HT_{1F} Receptor Agonists. There are two major classes of 5-HT_{1F} receptor agonists, triptans and flurobenzamides. Triptans are specific and selective 5-HT_1 receptor agonists that exhibit affinity for 5-HT_{1B}, 5-HT_{1D} and 5-HT_{1F} receptors (246). Triptans were specifically developed for the treatment of migraines. These five compounds exert their anti-migraine effects through the vasoconstriction of cranial vessels, inhibition of dual vasodilation by blocking the release of vasoactive neuropeptides, such as calcitonin gene-related peptide (CGRP) and blockade of nociceptive neurotransmission (248). It is noteworthy to mention that triptans have higher affinity for the 5-HT_{1B} and 5-HT_{1D}

receptors, owing to the vasoconstrictive effects of these compounds. Systematic vasoconstriction is a major limitation in the utilization of triptans as a therapeutic strategy, especially in the case of chronic treatment. Thus, specific 5-HT_{1F} receptor agonists were developed to augment migraine pain independent of vasoconstriction.

To this end, 4-fluoro-*N*-[3-(1-methyl-4-piperidinyl)-1*H*-indol-5-yl]benzamide (LY334370) and *N*-[(3*R*)-3-(dimethylamino)-2,3,4,9-tetrahydro-1*H*-carbazol-6-yl]-4-fluorobenzamide (LY344864) were developed and both of these compounds failed to induce vasoactive changes in the vasculature of human, bovine, rodent and rabbit (244, 249, 250). LY334370 and LY344864 are selective and efficacious agonists of the 5-HT_{1F} receptor with a reported pKd of 8.7and 8.2, respectively (251, 252). It is important to mention that LY334370 also has affinity for the 5-HT_{1A} receptor with a pKd of 7.8, while LY344864 binds with 100 fold greater affinity for the 5-HT_{1F} receptor than other 5-HT receptors (251, 252). Selective 5-HT_{1F} receptor stimulation is proposed to augment migraine pain by blocking migraine nociceptive transmission and preventing the release of glutamate, which is thought to play a role in migraine development (244, 246). In addition to these compounds, lasmiditan, the most recently developed selective 5-HT_{1F} receptor agonist, is currently in the final stage of clinical trials for migraine treatment, eliciting its effects through mechanisms similar to those of LY334370 and LY344864 (253, 254).

5-HT_{1F} Receptor in MB

Given the aforementioned role of serotonin receptors in modulating mitochondrial homeostasis, it is unsurprising that the 5-HT_{1F} selective agonists, LY334370 and LY344864 yielded positive results in our phenotypic screening for MB. Specifically, both compounds (1-100 nM) significantly increased FCCP-uncoupled oxygen consumption rates by 30-40% in RPTC at 24 hr (192). Comparatively, the non-selective serotonin receptor agonist, αm5-HT increased FCCP-uncoupled oxygen consumption rates by 20-

30% in RPTC (192), suggesting that both compounds are exhibiting FCCP-uncoupled oxygen consumption rates at the maximal potential for serotonin agonists.

To confirm that the observed increase in FCCP-uncoupled oxygen consumption rates were indeed a consequence of MB, gene expression of PGC-1α and respiratory chain components (COX1, ND6, NDUFB8), in addition to mtDNA copy number, were measured in RPTC treated with LY3344370 and LY344864. Interestingly, LY344864 increased gene expression of all endpoints, however, LY334370 only enhanced the gene expression of PGC-1α, COX1 and ND6 at 24 hr. In addition, LY334370 and LY344864 both increased protein expression of ATP Synthase β, COX1 and NDUFB8 in RPTC at 24 hr (192). These observed effects on MB were prevented by siRNA knockdown of the 5-HT_{1F} receptor, confirming that the mechanism by which LY334370 and LY344864 induce MB is 5-HT_{1F} receptor dependent (192). It is important to note that LY344864 was used to further characterize the role of the 5-HT_{1F} receptor as LY334370 was proven to be less efficacious for the induction of MB and demonstrated toxicity in larger animal species.

To determine whether MB was altered in the renal cortex because of LY344864 treatment, we assessed mRNA expression of renal cortical PGC-1α, COX1 and NDUFB8 in time course and dose dependent studies. A single dose of LY344864 (2 mg/kg) rapidly increased transcript levels of PGC-1α and COX1 (1 hr) followed by elevated NDUFB8 mRNA expression (8 hr) and mtDNA copy number (24 hr) in the renal cortex of mice (192). Taken together, these findings confirm the hypothesis that 5-HT_{1F} receptor stimulation induces MB in both primary RPTC and murine kidneys.

To assess the role of the 5-HT_{1F} receptor in MB and renal function under pathological conditions, mice subjected to bilateral renal ischemia were treated daily with saline vehicle or LY344864 over the course of 144 hr following surgery. I/R mice treated with

LY344864 showed accelerated recovery of renal function by decreased BUN levels from the initiation of treatment to near-control levels at the completion of treatment (192). Tubular injury was measured using renal cortical KIM-1 in both groups of I/R mice. As expected, KIM-1 protein expression was significantly reduced in LY344864-treated mice compared to mice that did not receive LY344864 (192). Treatment with LY344864 also promoted the recovery of renal cortical mtDNA copy number. Together, these findings demonstrate that the stimulation of 5-HT_{1F} receptor is a viable strategy to accelerate the recovery of renal function and tubular repair in the setting of AKI, and the 5-HT_{1F} receptor-dependent renal recovery is correlated with restoration of mtDNA copy number.

PROJECT GOALS

As discussed above, AKI is a severe disease characterized by a rapid loss in renal function that leads to substantial morbidity and mortality (7). The treatment options for AKI are currently limited to supportive measures and dialysis, indicating a tremendous need for the identification of novel targets for the development of effective therapeutics (69). Following renal I/R injury, persistent mitochondrial dysfunction and depletion of mitochondrial proteins correlate with sustained renal tubular injury and renal dysfunction (191). It has been further demonstrated that I/R injury is attenuated following the generation of new, functional mitochondria through the stimulation of MB (192, 193, 199). However, few pharmacological agents are known to induce MB. To this end, our laboratory has recently demonstrated that the 5-HT_{1F} receptor agonist LY344864 stimulated MB *in vitro* in RPTC and *in vivo* in healthy mouse kidneys. Additionally, LY344864 administration increased mtDNA copy number and improved renal function in mice subjected to I/R-induced AKI.

To our knowledge, these are the first evidence linking the 5-HT_{1F} receptor to mitochondria. Therefore, the first goal of this study was to identify the early signal transduction mechanisms by which LY344864 induces MB in primary RPTC. In order to address this objective, we utilized pharmacological inhibitors of known cellular mediators of MB. This aim focused on the eNOS/cGMP/PKG pathway, which has been implicated as a mitochondrial biogenic pathway. We also explored ERK1/2 as a potential negative regulator of LY344864-induced MB. Additionally, based on the classification of the 5-HT_{1F} receptor as a $G\alpha_{i/o}$-coupled GPCR, We examined the role of the $G\beta\gamma$ heterodimer signaling in LY344864-induced MB.

Another goal of this study was to determine the physiological role of the 5-HT_{1F} receptor in mitochondrial homeostasis and renal function. In order to address this aim, we

established a breeding colony of 5-HT_{1F} receptor wild-type (WT), heterozygous (HET) and homozygous (KO) mice. We hypothesized that renal cortical mitochondrial homeostasis is disrupted in the absence of the 5-HT_{1F} receptor and that this disruption impairs renal function. Additionally, mitochondrial homeostasis endpoints were explored in cardiac left ventricle as stimulation of the 5-HT_{1F} receptor has been shown to induce cardiac MB and the heart is a well-documented contributor and target of I/R-induced AKI. Since mitochondria are critical regulators of age-related pathologies, We also focused on mitochondrial homeostasis with respect to age in the 5-HT_{1F} receptor KO mouse, with an expectation of worsened outcomes over time.

The final aim of this study was to examine the role of 5-HT_{1F} receptor signaling on PGC-1α, mitochondrial homeostasis and function in I/R-induced AKI and the recovery of renal function thereafter. Based on considerable evidence demonstrating the renal protective effects induced by 5-HT_{1F} receptor stimulation I expected 5-HT_{1F} receptor KO mice to have worsened mitochondrial homeostasis and function and subsequently diminished recovery of renal function following I/R-induced AKI compared to WT controls. We hypothesized this aim would identify 5-HT_{1F} receptor dependent MB as an essential regulator of mitochondrial homeostasis and function for the proper recovery of renal function in the setting of I/R-induced AKI.

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CHAPTER TWO:

Identification of Dual Mechanisms Mediating 5-hydroxytryptamine Receptor 1F Induced MB

ABSTRACT

Our laboratory recently made the novel observation that 5-hydroxytryptamine 1F (5-HT_{1F}) receptor activation induces MB, the production of new, functional mitochondria, in *vitro* and *vivo*. We sought to determine the mechanism linking the 5-HT_{1F} receptor to MB in renal proximal tubule cells. Using LY344864, a selective 5-HT_{1F} receptor agonist, we determined that the 5-HT_{1F} receptor is coupled to Gαi/o and induces MB through Gβγ dependent activation of Akt, endothelial nitric oxide (eNOS), cyclic guanosine-monophosphate (cGMP), protein kinase G (PKG) and peroxisome proliferator-activated receptor gamma coactivator-1α (PGC-1α). We also report that the 5-HT_{1F} receptor signals through a second, Gβγ dependent pathway that is linked by Akt phosphorylation of Raf. In contrast to the activated Akt pathway, Raf phosphorylation reduced ERK1/2 and FOXO3a phosphorylation, suppressing an inhibitory MB pathway. These results demonstrate that the 5-HT_{1F} receptor regulates MB through Gβγ dependent dual mechanisms that activate a stimulatory MB pathway, Akt/eNOS/cGMP/PKG/PGC-1α, while simultaneously repressing an inhibitory MB pathway, Raf/MEK/ERK/FOXO3a. Novel mechanisms of MB provide the foundation for new chemicals that induce MB to treat acute and chronic organ injuries.

INTRODUCTION

In addition to its roles as a hormone and neurotransmitter in the central nervous system, 5-HT (serotonin, 5-hydroxytryptamine) mediates vascular contraction and relaxation, gastrointestinal motility, apoptosis and platelet aggregation through peripheral receptors (1-6). The biological roles of 5-HT are mediated by a family of G protein-coupled receptors (GPCR).Recently, our group revealed a novel role for 5-HT receptors in MB (MB), or generation of new, functional mitochondria (7-9).

MB is an attractive target for pharmacological intervention following acute organ injuries such as ischemia reperfusion (IR) injury (10, 11). Rapid and persistent loss of mitochondrial homeostasis is a major contributor to the pathology of IR-induced renal injury and IR suppresses peroxisome proliferator-activated receptor gamma coactivator-1α (PGC-1α), the master regulator of MB, and its downstream targets (10, 11). Increasing PGC-1α promotes the transcription of genes necessary for mitochondrial function during repair and restoration following oxidant injury in renal proximal tubule cells (RPTC) and IR-induced acute kidney injury (AKI) (12-15).

Despite the promise of MB as a therapeutic target, few nontoxic pharmacological agents stimulate PGC-1α expression and activity. Our laboratory developed a phenotypic assay to measure MB and identified several pharmacological targets that activate PGC-1α and induce MB, including the 5-HT_{1F} receptor (7, 16). The selective 5-HT_{1F} receptor agonist LY344864 is a potent and efficacious inducer of MB *in vitro* and *in vivo* as demonstrated by increased electron transport chain gene and protein levels (7). LY344864 exhibits high affinity for the 5-HT_{1F} receptor with a reported pKd of 8.2 and is ~100 times more selective for the 5-HT_{1F} receptor compared to 5- HT_{1A}, $_{1B}$, $_{1D}$, $_{1E}$ (17). Additionally, LY344864-induced 5-HT_{1F} receptor activation in a mouse model of IR- AKI restored mitochondrial DNA (mtDNA) copy number and accelerated recovery of renal function (7),

providing evidence that the 5-HT_{1F} receptor is a good therapeutic target to stimulate MB and promote recovery from acute organ failure. While we have identified a novel role for this receptor, however, the signaling mechanism of 5-HT_{1F} receptor-induced MB remains unknown. In this paper we identify the signaling pathways responsible for MB by 5-HT_{1F} receptor activation.

METHODS

Reagents

LY344864, pertussis toxin, gallein, LY294002, LNAME, ODQ, and KT5823 were purchased from Tocris (Ellisville, MO). GDC0068 was purchased from Selleckcem. (Houston, TX).

Isolation and Culture of Renal Proximal Tubule Cells and Oxygen Consumption

Female White New Zealand Rabbits (1.5-2.0 kg) were purchased from Charles River Laboratories (Wilmington, MA). RPTC were isolated using the iron perfusion method previously described (18). RPTC were plated and cultured in 96-well respiratory plates or 35-mm dishes in media previously described (7). Experiments were performed on the third or sixth day after plating when cells had formed a confluent monolayer. Oxygen consumption rate (OCR) of RPTC was measured using the Seahorse Bioscience XF-96 Extracellular Flux Analyzer as previously described (16). Each 96-well assay plate was treated with vehicle (DMSO, < 0.5%) or the experimental compounds. Basal OCR was measured before injection of carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP, 0.5μM) to measure the uncoupled OCR (FCCP-OCR), a marker of MB (16). All studies conducted were approved by the Institutional Animal Care and Use Committee at the Medical University and University of Arizona.

Analysis of Mitochondrial DNA Content

Mitochondrial DNA content was determined by quantitative real-time PCR analysis. Total DNA was isolated from RPTC using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) as described in the manufacturer's protocol. Extracted DNA was quantified and 5 ng was used for PCR. Relative mitochondrial DNA content was assessed by the mitochondrial encoded NADH Dehydrogenase 1 (ND1) and was

normalized to nuclear- encoded β- Actin. Primer sequences for ND1 and β- Actin were: 5'-TAGAACGCAAAATCTTAGGG- 3'; ND1 antisense: 5'-TGCTAGTGTGAGTGATAGGG-3'; β-Actin sense: 5'- GGGATGTTTGCTCCAACCAA-3'; and β-Actin antisense: 5'- GCGCTTTTGACTCAGGATTTAA-3'.

Protein Isolation, Immunoblot Analysis and Immunoprecipitation

Protein isolation and immunoblot analysis were performed as previously described (7). For immunoprecipitation experiments, protein (500 *μ*g) were pre- cleared by incubating with Pierce Protein A/G Plus Agarose beads for 30 min, then centrifuged at 14, 000 x g for 10 min at 4°C. The supernatant was collected and incubated with an anti-PGC-1α antibody (3 *μ*g) overnight at 4°C. Pierce Protein A/G Plus Agarose beads were washed and incubated with PGC-1α-protein lysates for 2 hr at 4°C. Lysates were then washed in immunoprecipitation buffer (25 mM Tris, 150 mM NaCl, pH 7.4) followed by centrifugation at 2,000 x g for 1 min. Laemmli buffer and β-mercaptoethanol (1:50) was added to collected supernatant and incubated at 95 °C for 5 min. Following a brief centrifugation, the supernatant was collected and analyzed by immunoblotting using antibodies against phosphoserine/threonine (1:1000) from Abcam (Cambridge, MA) and PGC-1α (1:1000) from EMD Millipore (Billerica, MA). Primary antibodies p-AKT Ser473 (1:000), total AKT (1:1000), p-eNOS Ser1177 (1:500), p-VASP Ser239 (1:1000), total VASP (1:1000), p-ERK p44/42 (1:1000), total ERK (1:1000), p-FOXO3a Ser294, and total FOXO3a were purchased from Cell Signaling Technologies (Danvers, MA), total eNOS (1:1000) was purchased from Abcam and GAPDH (1:10,000) from Fitzgerald (Acton, MA). Secondary antibodies include horseradish peroxidase- labeled anti rabbit and mouse from Abcam (Cambridge, MA).

cAMP and cGMP Enzyme-Linked Immunosorbent Assay

RPTC in 35-mm dishes were treated with DMSO or LY for 1 hr. RPTC were then harvested according to the manufacturer's protocol and cAMP or cGMP levels were measured using an ELISA kit (Cayman Chemical, Ann Arbor, MI). cAMP and cGMP values (pmol) were normalized to protein (mg) as quantified by a bicinchoninic acid assay followed by normalization to vehicle control for each biological replicate.

Statistical Analysis

Data are presented as the mean ± S.E.M. Single comparisons were performed using the Student's *t* test. Multiple comparisons were subjected to one-way analysis of variance followed by the Tukey's post hoc test, with $p < 0.05$ considered to be a statistically significant difference between means. Different subscripts indicate statistically differences. RPTC isolated from a single rabbit represented an individual experiment ($n = 1$) and were repeated until $n = 4\text{-}5$ was obtained.

RESULTS

5-HT_{1F} receptor agonism decreases cAMP formation and induces FCCP-uncoupled OCR and mtDNA Copy Number in a Gβγ-Akt-NOS-PKG dependent manner

Because the 5-HT_{1F} receptor has been reported to be negatively coupled to adenylyl cyclase via $G\alpha_{i/o}$ (19), we first sought to explore the roles of the $G\alpha_{i/o}$ and $G_{\beta\gamma}$ in RPTC (20). To verify that the renal 5-HT_{1F} receptor is a $G\alpha_i$-coupled GPCR, we measured cAMP in LY treated RPTC in the presence and absence of pertussis toxin (PT). PT catalyzes the ADP-ribosylation of the $G\alpha_{i/o}$ subunits locking the α subunits into an inactive state and inhibiting adenylate cyclase activity, leading to increased cellular cAMP (21). RPTC were pretreated with 100ng/ml PT or DMSO for 24 hr, followed by a 20 min exposure to 10 nM LY344864 or DMSO. A 10 nM concentration of LY344864 was previously determined to induce MB in RPTC (7). Treatment with PTX alone increased cAMP levels (Fig.1A). LY344864 reduced cAMP formation by 70% and this reduction was blocked by PT pretreatment (Fig. 2.1A), verifying that LY344864-induced 5-HT_{1F} receptor signaling in RPTC is mediated by $G\alpha_{i/o}$, PT-sensitive G proteins.

Our laboratory developed a high-throughput screening assay to assess MB by measuring FCCP-induced uncoupled oxygen consumption rates (FCCP-OCR) in RPTC, a marker of MB (16). Previous studies demonstrated that LY-induced FCCP-OCR resulted from increased mtDNA number and electron transport chain proteins at 24 hr (7). To understand the signaling pathways leading to increased FCCP-OCR, inhibitors of Gβγ and other pathways were analyzed for their ability to block LY344864-induced FCCP-OCR. RPTC were pretreated for 30 min with DMSO or gallein (Gal), GDC0068 (GDC), LNAME, ODQ, and KT5823 (KT), inhibitors of Gβγ, Akt, nitric oxide synthase (NOS), soluble guanylyl cyclase and protein kinase G (PKG), respectively (22-25), and then exposed to DMSO or LY344864 for 24 hr. Pretreatment with these inhibitors prevented LY-induced FCCP-OCR (Fig. 2.1B). In addition, pretreatment with gallein,

GDC, LNAME, ODQ and KT prevented LY344864-induced mtDNA copy number (Fig. 2.1C), suggesting that Gβγ, Akt, NOS, cGMP, and PKG are key regulators of 5-HT_{1F} receptor induced MB pathway.

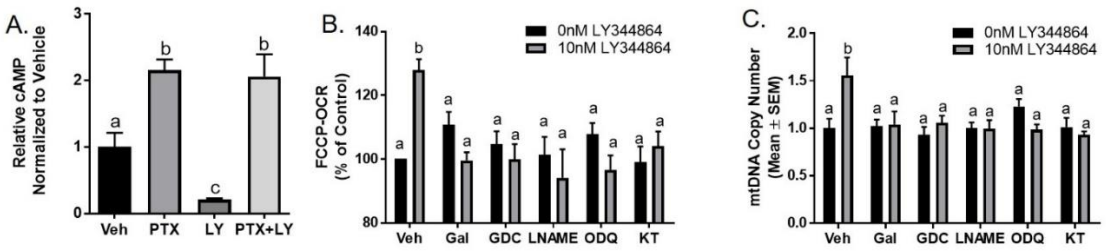

Figure 2.1: 5-HT$_{1F}$ receptor agonism decreases cAMP formation and induces FCCP-uncoupled OCR and mtDNA Copy Number in a Gβγ-Akt-NOS-PKG dependent manner. cAMP levels were measured by ELISA after a 20 min treatment with <0.5% DMSO, 10 nM LY344864 or a 24 hr pretreatment of pertussis toxin (100ng/mL) followed by DMSO or LY (A). RTPC were pretreated with DMSO or pharmacological inhibitors, gallein (Gal,100 nM), GDC0068 (GDC,100 nM), LNAME (10 µM), ODQ (100 nM), or KT5823 (KT, 100 nM) for 30 min. DMSO or LY344864 were then added and FCCP-OCR uncoupled mitochondrial respiration was measured using Seahorse XF 96 analyzer 24 hr later (B). RTPC were pretreated with DMSO or pharmacological inhibitors, Gal, GDC, LNAME, ODQ or KT for 30 min followed by 24 hr exposure of DMSO or LY and mtDNA copy number was assessed (C). Data are reported as mean ± SEM, n =4-5. Bar with different superscripts are significantly different from one another ($p < 0.05$).

LY344864 induced Akt and eNOS phosphorylation is blocked by inhibitors of Gβγ, PI3K and Akt

To examine Akt phosphorylation following LY344864 treatment, RPTC were treated with LY344864 or DMSO for 15 min, 30 min, 1, 2, and 3 hr. Immunoblot analyses revealed a 2-fold increase in p-Akt at Ser473 at 15 min and a 1.5-fold increase at 30 min (Fig. 2.2A, B). RPTC were pretreated with gallein (100 nM, 30 min) and exposed to LY344864 for 15 min. Immunoblot analyses revealed that gallein inhibited LY344864-induced Akt phosphorylation (Fig. 2.2C, D). Because Akt is a substrate of PI3K, RPTC were pretreated with the PI3K inhibitor LY294002 (10 μM, 30 min) and then treated with LY344864. Blockade of PI3K inhibited Akt phosphorylation after treatment (Fig. 2.2E, F). Next, we examined eNOS phosphorylation at Ser1177, a direct phosphorylation target of Akt. RPTC were treated with DMSO or LY344864 for 1, 2, 3, and 4 hr. eNOS phosphorylation was elevated 2.5- and 3-fold at 1 and 2 hr, respectively (Fig. 2.2G, H). To confirm that Gβγ and Akt are upstream of eNOS phosphorylation, RPTC were pretreated with gallein and GDC (100 nM, 30 min), then exposed to LY344864 for 1 hr (Fig. 2.2I, L). Inhibiting Gβγ and Akt also prevented LY344864-induced eNOS phosphorylation. These data demonstrate that LY344864 activates the Gβγ-PI3K-Akt-eNOS pathway.

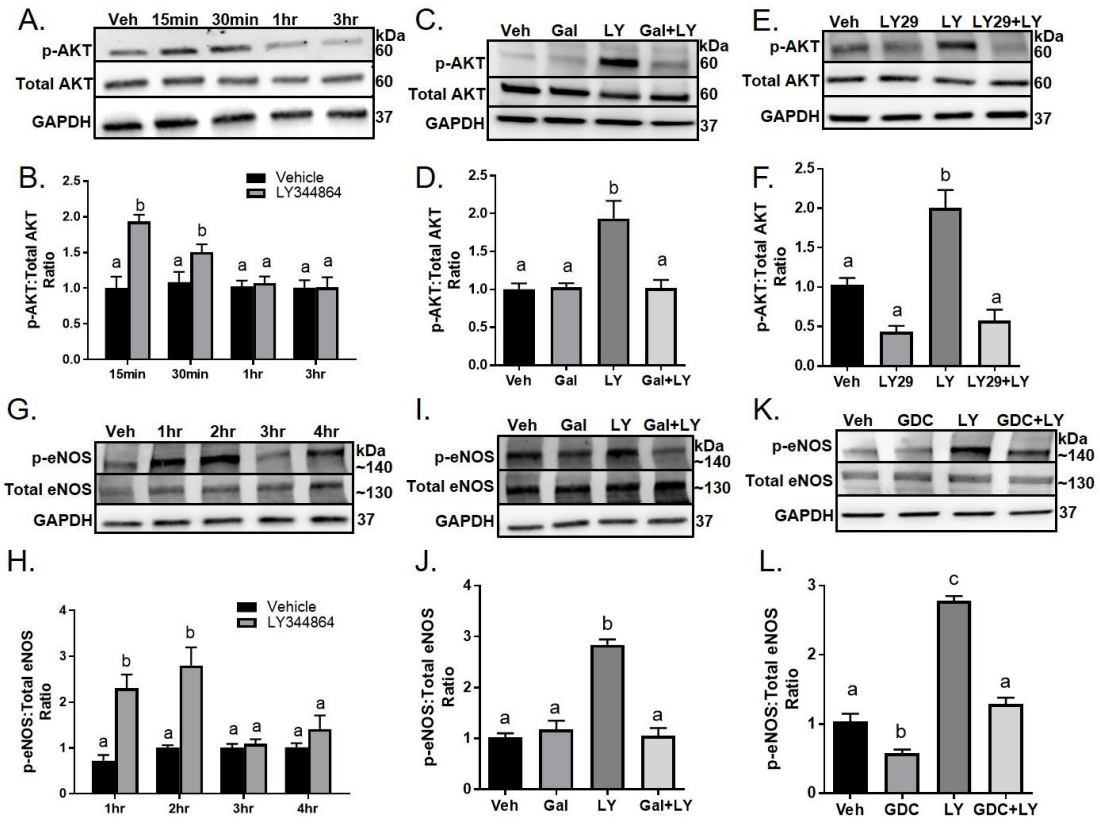

Figure 2.2: LY344864 induced Akt and eNOS phosphorylation is blocked by inhibitors of Gβγ, PI3K and Akt. Phosphorylated Akt (Ser473) was measured by immunoblot analysis 15 min, 30 min, 1 hr, or 3 hr after treatment with DMSO or LY344864 (A, B), after 30 min pretreatment with DMSO or gallein (Gal, 100 nM) followed by exposure to DMSO or LY344864 for 15 min (C, D), and after 30 min pretreatment with DMSO or LY29004 (LY29, 10 µM) followed by exposure to DMSO or LY344864 for 15 min (E, F). Phosphorylated eNOS (Ser1177) was measured by immunoblot analysis after 1, 2, 3, or 4 hr treatment with DMSO or LY344864 (G, H), after 30 min treatment with DMSO or Gal (100 nM) followed by exposure to DMSO or LY for 1 hr (I, J), and after 30 min pretreatment with DMSO or GDC0068 (GDC, 100nM) followed by exposure to DMSO or LY344864 for 1 hr (K, L). Data are reported as mean ± SEM, n≥5. Bars with different superscripts are significantly different from one another ($p < 0.05$).

LY344864 increases cGMP formation and cGMP- and PKG-dependent VASP phosphorylation

Numerous reports demonstrate that increased PGC-1α expression and other markers of MB in tissues, including the kidney, are elevated through an increase in cGMP production (26-28). To determine if cGMP is involved in LY344864-induced MB, we measured cGMP following LY344864 treatment. RPTC were treated with the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX, 100 μM), and DMSO or LY344864. LY344864 elicited a transient 1.6-fold increase in cGMP at 1 hr that returned to baseline levels at 2 hr (Fig. 2.3A). The formation of cGMP induces a conformational change in cGMP-dependent protein kinase (PKG) allowing activation of this serine/threonine protein kinase and the phosphorylation of substrate proteins (29). Vasodilator-stimulated phosphoprotein (VASP), a marker of PKG activation, was assessed to determine the role of PKG in LY344864-induced MB signaling (30). RPTC expressed the PKG splice variant PKGI which preferentially phosphorylates VASP at Ser239 (31). LY344864 increased VASP phosphorylation 1.8- and 2-fold at 1and 2 hr, respectively (Fig. 2.3B, C). To determine if cGMP production and PKG activation are responsible for increased VASP phosphorylation, RPTC were pretreated with ODQ (100 nM, 30 min) or KT (100 nM, 30 min) and then exposed to LY344864 for 1 hr. LY344864-induced p-VASP upregulation was inhibited by blockade of cGMP production (ODQ) and PKG activation (KT) (Fig. 2.3D, G). Taken together these findings reveal agonist stimulation of the 5-HT_{1F} receptor mediates Gβγ-PI3K-AKT-eNOS-cGMP-PKG-VASP signaling.

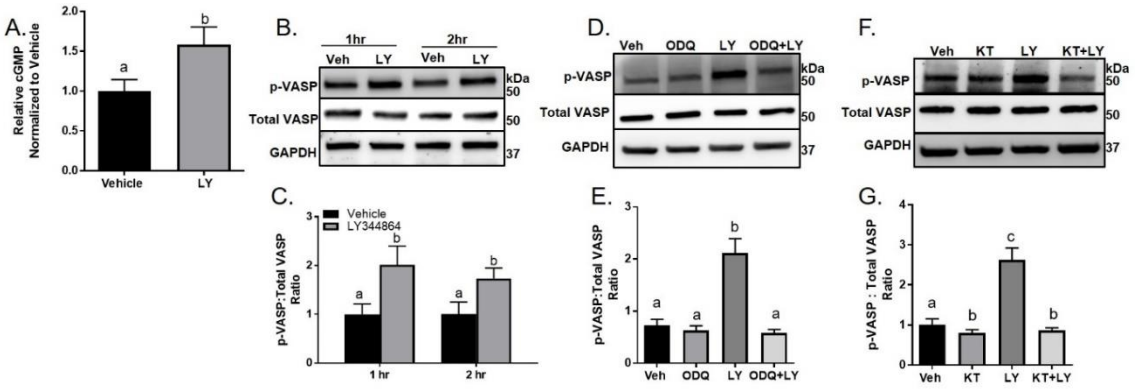

Figure 2.3: LY344864 increases cGMP formation and cGMP- and PKG- dependent VASP phosphorylation. cGMP levels were measured by ELISA after 1 hr treatment with DMSO or LY344864 in the presence of 3-isobutyl-1-methylxanthine (IBMX, 100 µM, A). Phosphorylated VASP (Ser239) was measured by immunoblot analysis 1 or 2 hr after treatment with DMSO or LY344864 (B, C), after 30 min treatment with DMSO or ODQ (100 nM) followed by exposure to DMSO or LY344864 for 1 hr (D, E), and after 30 min treatment with DMSO or KT5923 (KT, 100 nM) followed by exposure to DMSO or LY344864 for 1 hr (F, G). Data are reported as mean ± SEM, n ≥ 5. Bars with different superscripts are significantly different from one another ($p < 0.05$).

LY344864 increases phosphorylated serine/threonine residues on PGC-1α

PGC-1α is regulated by numerous posttranslational modifications such as phosphorylation, methylation, and acetylation (10, 32-34). Since we elucidated a number of activated kinases in the 5-HT_{1F} receptor signaling pathway, we examined the phosphorylation of PGC-1α in RPTC exposed to LY344864 or DMSO for 2 hr. PGC-1α was immunoprecipitated from RPTC and subjected to immunoblot analysis with antibodies against phosphoserine/threonine residues and PGC-1α. The ratio of phosphorylated serine/threonine to total PGC-1α was elevated 2.8-fold and this increase was attenuated following Akt and PKG inhibition (Fig. 2.4A, B), suggesting posttranslational activation during 5-HT_{1F} receptor signaling in RPTC is dependent upon Akt and PKG activity.

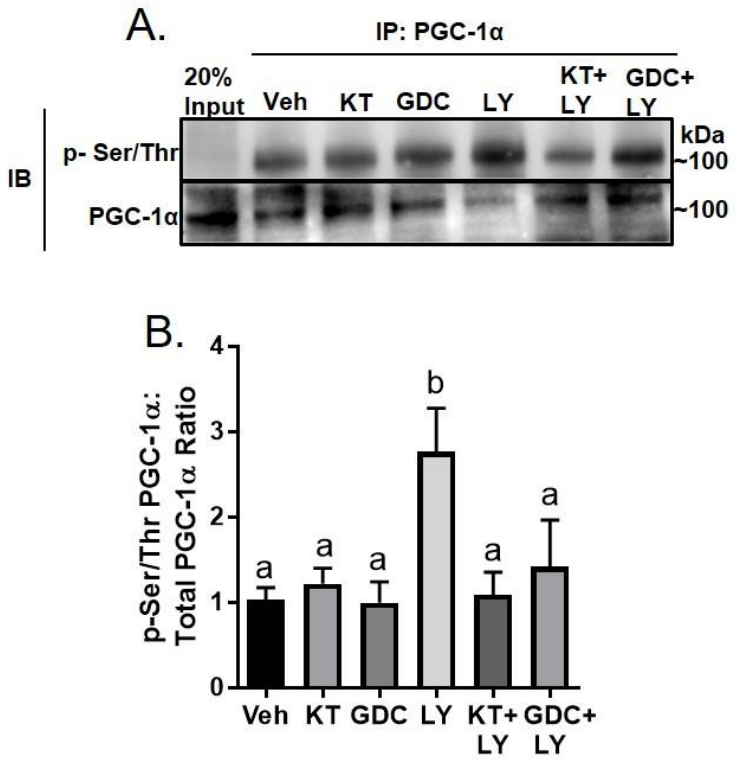

Figure 3.4: LY344864 increases phosphorylated serine/threonine residues on PGC-1α. Phosphorylated serine/threonine residues were measured following immunoprecipitation of PGC-1α by immunoblot analysis after 30 min treatment with DMSO, KT (100nM) or GDC (100nM) followed by exposure to DMSO or LY344864 for 2 hr treatment (A, B). Total PGC-1α expression was measured to verify presence of PGC-1α protein following immunoprecipitation and equal protein input. Twenty percent of total protein lysate was used to verify immunoprecipitation of PGC-1α was successful. Data are reported as mean ± SEM, n=5. Bars with different superscripts are significantly different from one another ($p < 0.05$).

LY344864 reduces ERK phosphorylation and inhibitors of Gβγ and Akt prevent the reduction in ERK phosphorylation

ERK1/2 signaling has been implicated as a negative regulator of MB (35). We analyzed the phosphorylation of ERK1/2 in RPTC at 1, 2, and 3 hr after LY treatment. ERK1/2 phosphorylation decreased 50% at 1 hr (Fig. 2.5A, B). We hypothesized that this reduction was also mediated by Gβγ signaling. RPTC were pretreated with DMSO and gallein (100 nM, 30 min), followed by 1 hr exposure of DMSO or LY344864. Blockade of Gβγ prevented LY344864-mediated decrease in ERK1/2 phosphorylation elevating ERK1/2 phosphorylation above vehicle levels (Fig. 2.5C, D).

To investigate cross-talk between LY344864-mediated Akt and ERK signaling, RPTC were pretreated with GDC (100nM, 30 min) and then DMSO or LY. Akt inhibition prevented decreased LY344864-mediated ERK1/2 phosphorylation and increased ERK1/2 phosphorylation compared to vehicle (Fig. 2.5E, F). RPTC were also pretreated with LNAME (10 µM, 30 min), prior to 1 hr treatment with LY344864 to determine if NOS is involved in modulating p-ERK1/2. ERK1/2 phosphorylation was unchanged compared to LY344864-treated RPTC, indicating that NOS was not responsible for reduced ERK1/2 phosphorylation (Fig. 2.5G, H). It is important to note that gallein and GDC alone increased ERK1/2 phosphorylation by 2.5- and 2-fold in RPTC, respectively, suggesting that inhibition of Gβγ-AKT modulates ERK1/2 under physiological conditions, strengthening the premise that the AKT and ERK1/2 pathways collaborate to maintain cellular processes in RPTC. Collectively, these data reveal that Gβγ and Akt activation is upstream of the suppression of ERK1/2 phosphorylation following LY344864 treatment in RPTC.

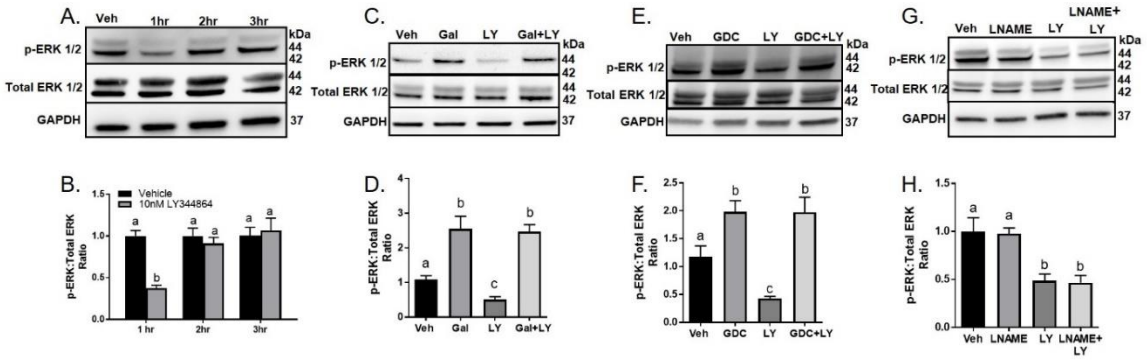

Figure 2.5: LY344864 reduces ERK phosphorylation and inhibitors of Gβγ and Akt prevent the reduction in ERK phosphorylation. Phosphorylated ERK (p44/42) was measured by immunoblot analysis 1, 2, or 3 hr after treatment with DMSO or LY344864 (A, B), after 1 hr treatment with DMSO or gallein (Gal,100 nM) followed by exposure to DMSO or LY344864 for 1 hr (C, D), after 1 hr treatment with DMSO or GDC0068 (GDC,100 nM) followed by exposure to DMSO or LY344864 for 1 hr (E, F), and after 1 hr pretreatment with DMSO or LNAME (10 μM) followed by exposure to DMSO or LY344864 for 1 hr (G, H). Data are reported as mean ± SEM, n ≥ 5. Bars with different superscripts are significantly different from one another ($p < 0.05$).

LY344864 induced c-raf phosphorylation at site Ser259 and is Akt-dependent

c-Raf is known to regulate the activity of the ERK1/2 signaling cascade. Typically, c-raf phosphorylation of Ser338 activates the MEK-ERK1/2 pathway, while phosphorylation of site Ser259 is inhibitory (36, 37). c-Raf Ser259 is a target of active Akt and there is evidence that Raf-MEK-ERK and PI3K-Akt pathways cross-talk at the level of raf and Akt (37, 38). We detected phosphorylation of both Ser259 and Ser338 in DMSO and LY344864-treated RPTC for 15 and 30 min. Ser259 phosphorylation was elevated 1.5-fold at 30 min in the presence of LY344864, while Ser338 phosphorylation remained at control levels (Fig. 2.6A-C). Additionally, GDC (100 nM, 30 min) pretreatment, followed by 30 min LY344864 exposure inhibited Ser259 phosphorylation (Fig. 2.6D, E). In summary, LY344864 exposure resulted in Akt-dependent c-raf inhibition, further evidence that ERK1/2 inhibition is regulated by Akt.

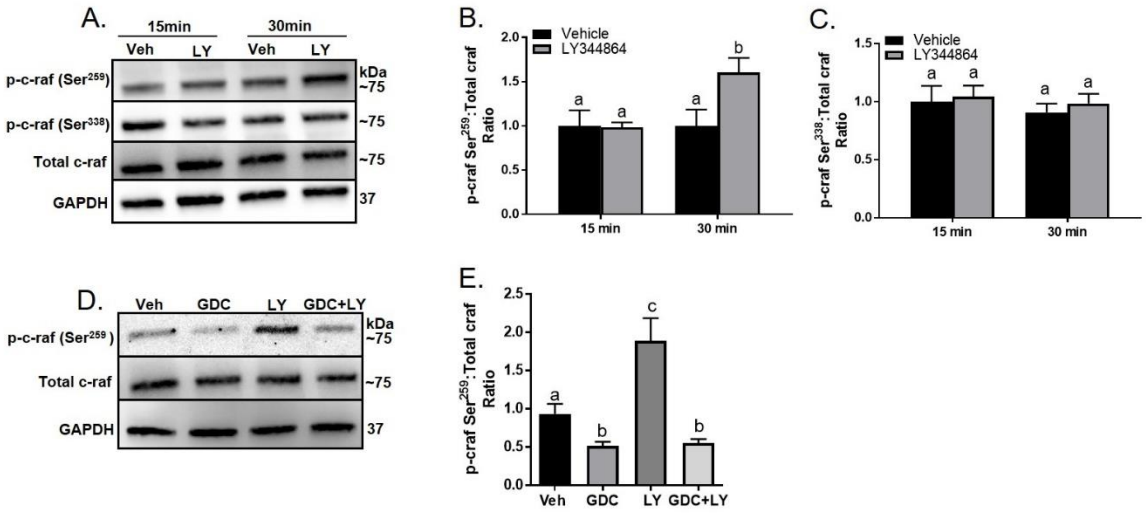

Figure 2.6: LY344864 induced c-raf phosphorylation at site Ser259 and is Akt-dependent. Phosphorylated c-raf was measured by immunoblot analysis 15 or 30 min after treatment with DMSO or LY344864 (A). Densitometry analysis of Ser259 (B) and Ser338 (C) phosphorylation. Phosphorylated c-raf (Ser259) was measured by immunoblot analysis after 30 min treatment with DMSO or GDC0068 (GDC,100 nM) followed by exposure to DMSO or LY for 15 min (D, E). Data are reported as mean ± SEM, n ≥ 5. Bars with different superscripts are significantly different from one another ($p < 0.05$).

LY344864 reduces FOXO3a phosphorylation

We recently reported that ERK1/2 inhibition reduces phosphorylation of FOXO3a, allowing for increased nuclear FOXO3a to activate transcription of genes including PGC-1α (35). We observed that FOXO3a phosphorylation at Ser294 was reduced 40% by LY344864 at 2 hr (Fig. 2.7), suggesting that suppressed ERK1/2 phosphorylation up regulates PGC-1α through FOXO3a.

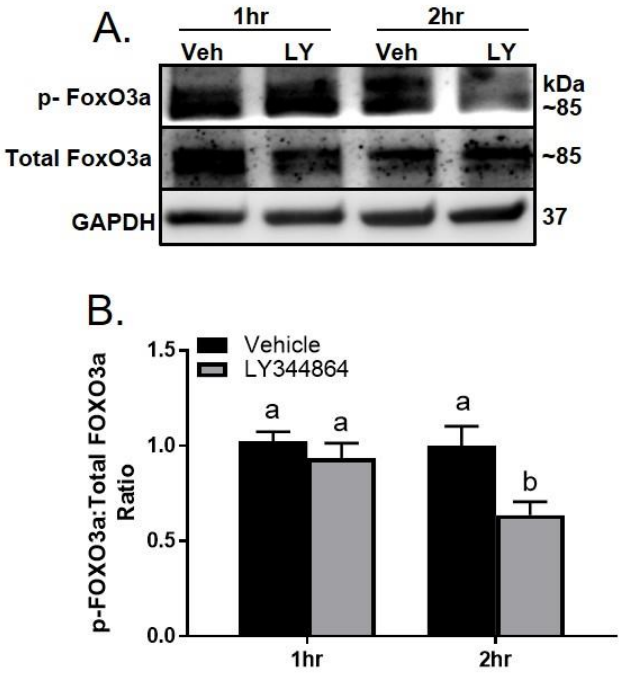

Figure 2.7: LY344864 reduces FOXO3a phosphorylation. Phosphorylated FOXO3a (Ser294) was measured by immunoblot analysis 1 or 2 hr after treatment with DMSO or LY344864. Data are reported as mean ± SEM, n ≥ 5.Bars with different superscripts are significantly different from one another ($p < 0.05$).

DISCUSSION

Classically, the 5-HT_{1F} receptor has been characterized as a mediator of pain without vasoconstriction, which led to the development of $5HT_{1F}$ agonists such as LY344864 for the treatment of migraines. However, the biological roles of peripherally expressed 5-HT_{1F} receptors have been understudied. We recently observed that LY344864 induces MB and accelerates recovery of renal function following IR-AKI in mice (7). The goal of this study was to elucidate the signaling mechanism connecting the 5-HT_{1F} receptor to MB. Utilizing a high-throughput MB screening assay and immunoblot analyses, we determined that Gβγ, Akt, NOS, cGMP, and PKG are crucial components in MB following 5-HT_{1F} receptor stimulation. In addition, a second, parallel pathway was identified that negatively regulates PGC-1α and MB through ERK and FOXO3a phosphorylation. This is the first study to report that Gβγ initiates MB through dual mechanisms, increasing Akt/eNOS/cGMP/PKG/PGC-1α and decreasing Raf/MEK/ERK/FOXO3a pathways (Fig.2.8).

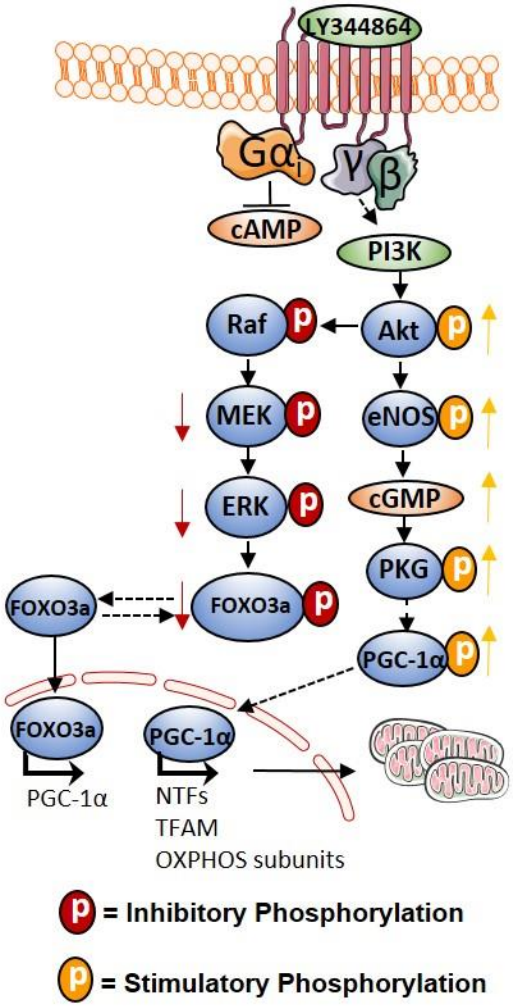

Figure 2.8: Proposed 5-HT$_{1F}$ receptor mediated MB pathway proceeds through dual mechanisms dependent upon Gβγ in RPTC. Gβγ initiates Akt phosphorylation and the subsequent phosphorylation of eNOS, both in a PI3K dependent manner. LY344864 increases cGMP production, induces PKG activation and PGC-1α phosphorylation in RPTC. Simultaneously, Raf phosphorylation reduces ERK1/2 and FOXO3a phosphorylation, also a Gβγ dependent process. Reduced FOXO3a phosphorylation promotes nuclear translocation of FOXO3a for transcription of genes such as PGC-1α. Orange phosphorylation sites indicates activation of downstream effectors. Red phosphorylation sites indicate reduction of downstream effectors. PI3K: phosphoinositide-3-kinase, Akt: protein kinase B, eNOS: endothelial nitric oxide synthase, cGMP: cyclic guanosine monophosphate, PKG: protein kinase G, PGC-1α: peroxisome proliferator- activated receptor gamma coactivaor-1α, Raf: rapidly accelerated fibrosarcoma, ERK: extracellular signal- regulated kinase, FOXO3a: forkhead box O3, MB: MB

Following ligand binding to the Gαi-coupled GPCR, the Gαi and Gβγ disassociate and activate downstream signaling. Gβγ has been shown to activate a variety of signal transduction pathways (39-42). Through the use of gallein, we demonstrated that Gβγ activates the PI3K-Akt pathway in RPTC following 5-HT_{1F} receptor stimulation. By recruiting the PI3K regulatory subunits p101 to the membrane, Gβγ is reported to be a direct activator of GPCR-induced PI3K activity. Gβγ has also been previously linked to Akt phosphorylation through PI3K stimulation (43).

This study not only elucidated the signaling mechanism of 5-HT_{1F} receptor-induced MB in RPTC, but also the duration of this signaling, which is crucial in the roles of Akt and NO. Specifically, chronic activation of Akt and NO has been linked to defective mitochondrial function and mitophagy (44-46). We demonstrate that Akt and eNOS are sequentially and transiently activated to produce MB as opposed to the oxidative stress that can occur due to prolonged NO production (47). NO is a key molecule in PGC-1α regulation predominantly through induction of soluble guanylate cyclase and cGMP (15). Downstream effectors of cGMP, such as PKG have also been linked to PGC-1α expression and activity (48, 49). Though the role of PKG in 5-HT_{1F} receptor induced MB remains unclear, we demonstrated that PKG is upstream of LY- induced PGC-1α phosphorylation. Further studies will elucidate the direct link between PKG and PGC-1α phosphorylation. One possibility is that the PKG substrate, p38 MAPK, is directly phosphorylating PGC-1α and increasing its activity. Several studies have reported p38 MAPK to phosphorylate and activate PGC-1α, specifically by disrupting the interaction between PGC-1α and the co-repressor p160MBP as observed in myoblasts (32, 49, 50). PGC-1α is also a substrate for a number of other kinases that regulates its activity. AMP-activated protein kinase (AMPK) phosphorylates and activates PGC-1α as well as increases its transcription (51), however, we did not observe any changes in AMPK

phosphorylation following acute or chronic 5-HT_{1F} receptor stimulation (data not shown). Furthermore, Akt substrates such as GSK3β and mTOR regulate PGC-1α activity (51-53), but the phosphorylation of these substrates were unchanged directly following Akt activation (data not shown). Collectively, these findings implicate the Akt/eNOS/cGMP/PKG signaling axis as a critical mechanism for LY- induced MB.

Interestingly, parallel to the activated Akt/eNOS/cGMP/PKG pathway, we observed decreased ERK/FOXO3a signaling. Collier et al. recently demonstrated that reduced FOXO3a phosphorylation leads to increased nuclear FOXO3a expression that ultimately increased PGC-1α (35). We determined that both Gβγ and Akt mediate LY344864 reduction of p-ERK1/2 and p-FOXO3a. Inhibition of Gβγ and Akt resulted in increased ERK1/2 phosphorylation, while activation of Gβγ and Akt following LY344864 treatment reduced ERK1/2 phosphorylation. These exciting results support the hypothesis that there is cross-talk between Raf/MEK/ERK and PI3K/Akt pathways in MB. Cross-talk between Raf/MEK/ERK and PI3K/Akt pathways has been reported in numerous cell types including renal cells (37, 54-56). Consistent with other findings, we determined the inhibitory 14-3-3 binding site on c-raf (Serine259) is directly phosphorylated by Akt, leading to reduced ERK1/2 signaling in RPTC treated with LY344864 (37).

Our group and others have demonstrated persistent disruption of mitochondrial homeostasis and inhibition of MB in myocardial infarction, spinal cord injury, stroke, drug- induced toxicities, and AKI (57-61). Restoration of mitochondrial number and function are necessary for normal cell and tissue function, and is critical in ATP-dependent repair processes for the recovery of organ function. Despite strong evidence supporting mitochondria as a therapeutic target, there are very few drugs available to promote mitochondrial function or MB. Many of these available agents lack specificity, potency, or have toxic effects.

Our laboratory has identified potent inducers of MB that act on different targets. For example, the 5-HT_{2A} receptor agonist, NBOH-2C-CN and the β_2-adrenergic receptor agonist formoterol, are potent inducers of PGC-1α and MB *in vitro* and *in vivo* (8, 9, 14). Additionally, specific inhibition of phosphodiesterases 3 and 5 increased cGMP to induce MB and accelerate the recovery of renal function following AKI (28). These studies provide evidence that induction of MB to stimulate repair and recovery of organ dysfunction is an effective approach to treat a variety of acute and chronic disease.

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CHAPTER THREE:

The 5-hydroxytryptamine Receptor 1F Regulates Mitochondrial Homeostasis in Kidney and Heart

ABSTRACT

Our laboratory previously reported that agonists of the 5-hydoxytryptamine 1F (5-HT1F) receptor induces renal and cardiac MB (MB). In addition, we reported that stimulation of the 5-HT_{1F} receptor following ischemia/reperfusion (I/R)-induced acute kidney injury (AKI) accelerated the recovery of renal function in mice. The goal of this study was to examine the contribution of the 5-HT_{1F} receptor in the regulation of renal and cardiac mitochondrial homeostasis and renal function in naïve and injured mice. While 5-HT_{1F} receptor knockout (KO) mice were healthy and fertile and did not exhibit renal dysfunction, renal MB and mitochondrial fission gene expression increased and heart MB and mitochondrial fission gene expression decreased at 10 weeks of age. Finally, 5-HT_{1F} receptor KO mice exhibited greater proximal tubular injury and diminished renal recovery after I/R-induced AKI compared to wild-type mice. These findings were also associated with the persistent suppression of renal cortical MB, and elevated oxidative stress markers. In summary, the 5-HT_{1F} receptor is a component of physiological MB regulation in renal and cardiac tissues, its absence potentiates renal injury and recovery, and it remains a promising target of MB and renal injury.

INTRODUCTION

Mitochondrial dysfunction is a hallmark pathophysiological mediator of many acute and chronic diseases. As such, loss of ATP-dependent cellular functions and increased reactive oxygen species propagate injury and subsequent tissue and organ dysfunction (1, 2). Specifically, our laboratory demonstrated that persistent mitochondrial dysfunction and suppression of MB corresponds to worsened renal function after I/R-induced AKI and folic acid-induced AKI (3-5).

MB has received increasing attention as a therapeutic target to promote restoration of normal cellular and organ function, and several studies have reported pharmacological induction of MB as a treatment for acute organ injury and cardiovascular disease (6-9). MB is defined as the dynamic process of generating new, functional mitochondria and is a highly regulated process controlled by the central mediator and transcriptional co-activator peroxisome proliferator-activated receptor α coactivator-1α (PGC-1α) (10). Several groups have developed strategies designed to increase the expression and activity of PGC-1α for the subsequent induction of MB, as evidenced by increased mitochondrial number and function (2, 11, 12). For example, we reported that a 5-hydroxytryptamine 1F (5-HT_{1F}) receptor agonist LY344864 induced MB *in vitro* and *in vivo*, restored MB, and accelerated the recovery of renal function following I/R-induced AKI in mice. (5). Furthermore, knockdown of the 5-HT_{1F} receptor in renal proximal tubular cells resulted in decreased mitochondrial proteins, suggesting that the 5-HT_{1F} receptor may be regulating MB under basal conditions. In addition to the renal MB findings, 5-HT_{1F} receptor stimulation also induced cardiac gene expression of nuclear and mitochondrial encoded electron transport chain (ETC) proteins and mitochondrial DNA (mtDNA) content in mice (5). These findings implicate the 5-HT_{1F} receptor as a potential regulator of MB in kidney and heart.

Based on these findings, we utilized a recently developed 5-HT_{1F} receptor knockout (KO) mouse to further understand the contribution of the 5-HT_{1F} receptor in the regulation of renal and cardiac mitochondrial homeostasis in young and aged mice. In addition, we tested the hypothesis that the absence of the 5-HT_{1F} receptor potentiates AKI in mice.

METHODS

I/R-induced AKI Model

Male and female 5-HT_{1F} receptor KO mice (B6N(Cg)-*Htr1ftm1.1(KOMP)Vlcg*/J, stock no. 024269) were age-matched wild-type female and male C57BL/6NJ (WT, stock no. 005304) purchased from the Jackson Laboratory (Bar Harbor, ME). A 5-HT_{1F} receptor KO breeding colony was generated from heterozygous mutants and were housed in temperature-controlled conditions under a light/dark photocycle with unrestricted food and water supplied *ad libitum*. Male and female mice were weighed periodically. Ten and 26 week old mice were chosen to evaluate age-related changes in 5-HT_{1F} receptor KO mice.

Ten-week old male WT and KO mice underwent I/R surgery using bilateral renal pedicle clamping for 18 min as described previously (13). Briefly, the renal artery and vein were isolated and blood flow was occluded with a vascular clamp for 18 min while body temperature was maintained at 36-37^{o}C. Sham mice were treated the same as I/R mice without clamping of the renal pedicles. Mice were euthanized 24 hr after surgery, and blood and kidneys (flash frozen in liquid nitrogen) were collected for analysis. Studies were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All protocols were approved by the Institutional Animal Care and Use Committee at the Medical University of South Carolina and the University of Arizona, and all efforts were made to minimize animal suffering.

Agarose Gel Electrophoresis

Genomic DNA was isolated from mice tail clippings using Viagen Direct PCR DNA Lysis Reagent (Los Angeles, CA) containing 0.4 mg/ml Proteinase K. PCR amplification was

performed using Promega PCR Master Mix (Madison, WI). PCR products were separated on a 2% agarose gel in 1x TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA) and 0.5 µg/mL ethidium bromide. Gels were visualized using the GE ImageQuant LAS4000 (GE Life Sciences, Pittsburgh, PA). Primer pairs annealing with the WT allele and the mutant allele were obtained from Jackson Laboratory and are listed in Table 3.1.

Complete Blood Count, Blood Urea Nitrogen (BUN) and Serum Creatinine Evaluation

Following euthanasia by isoflurane inhalation, peripheral blood was collected via cardiac puncture using EDTA as an anticoagulant and complete blood counts (CBC) were obtained using a HEMAVET Multispecies Hematology Analyzer. Blood was collected via retro-orbital bleed following isoflurane inhalation. BUN was determined using the QuantiChrom Urea Assay kit (BioAssay Systems, Hayward, CA) and serum creatinine was determined using the Creatinine Enzymatic Reagent Kit (Pointe Scientific, Canton, MI) based on the manufacturer's directions.

Protein Isolation and Immunoblot Analysis

Mouse kidney cortex and left ventricular cardiac tissue was homogenized in protein lysis buffer (1% Triton X 100, 150 mM NaCl, 10 mM Tris-HCl, pH 7.4; 1 mM EDTA; 1 mM EGTA; 2 mM sodium orthovanadate; 0.2 mM phenylmethylsulfonylfluoride; 1 mM HEPES pH 7.6; 1 µg/ml leupeptin; and 1 µg/ml aprotinin) using a polytron homogenizer. The homogenate was stored on ice for 10 min and then centrifuged at 7,500 *g* for 5 min at 4°C. The supernatant was collected and protein determined using a bicinchoninic acid kit (Sigma Chemical Co, St. Louis, MO) with bovine serum albumin as the standard. Proteins (50 µg) were separated on 4–20% gradient sodium dodecyl sulfate polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were blocked in 5% dried milk in TBST (0.1% Tween 20 in 1× TBS) for 1 hr and incubated

with antibodies for kidney injury molecule-1 (KIM-1) (1:1000; R&D systems, Minneapolis, MN), neutrophil gelatinase-associated lipocalin (NGAL), PGC-1α, TFAM, COX1, NDUFS1, 4-HNE and SOD2 (1:1000, Abcam, Cambridge, MA), overnight at 4°C. After incubation for 2 h at room temperature with donkey anti-goat secondary antibody (1:2000; Santa Cruz, Dallas, TX),goat anti-rabbit (1: 20000; Abcam) or rabbit anti-mouse (1:20000, Abcam) conjugated with horseradish peroxidase before visualization using enhanced chemiluminescence (Thermo Scientific, Waltham, MA) and the GE ImageQuant LAS4000 (GE Life Sciences, Pittsburgh, PA).Optical density was determined using the National Institutes of Health ImageJ software (version 1.46).Each target protein was normalized to β-Actin loading control.

Quantitative Real-Time Polymerase Chain Reaction Analysis of mRNA Expression

Total RNA was isolated from renal cortical and left ventricle cardiac tissue with TRIzol reagent (Life Technologies, Grand Island, NY). The iScript Advanced cDNA Synthesis Kit for quantitative real-time polymerase chain reaction qRT-PCR (Bio-Rad Laboratories, Hercules, CA) was used to produce a cDNA library from 1 μg total RNA according to the manufacturer's protocol. For human experiments, we obtained human renal cDNA from Takara Bio USA, Mountain View, CA. Human cardiac samples were obtained from BioChain Institute, Inc. We performed qRT-PCR with the generated cDNA using the SsoAdvanced Universal SYBR Green Supermix reagent (Bio-Rad Laboratories). The relative mRNA expression of all genes was determined by the $2^{-\Delta\Delta Ct}$ method, and β-actin was used as a reference gene for normalization. Primer pairs used for PCR are listed in Table 3.1.

Analysis of Mitochondrial DNA Content

Total DNA was isolated from the renal cortex and left ventricle cardiac tissue using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) as described by manufacturer's protocol. Extracted DNA was quantified and 5 ng was used for qRT-PCR. Relative DNA content was assessed by the mitochondrial-encoded NADH dehydrogenase subunit 1 (ND1) and was normalized to nuclear-encoded β-actin. Primer sequences are listed in Table 3.1.

Determination of ATP Content

Renal cortical ATP was measured using the ATP Assay Kit (Abcam). Briefly, mouse renal cortex was homogenized in ice-cold 2M perchloric acid using a polytron homogenizer. Homogenate was stored on ice for 45 min and then centrifuged at 13,000 x g for 2 min at 4°C. The supernatant was collected and diluted in the ATP Assay buffer. For each sample, pH was measured and adjusted to equal 6.5-8 using 0.1M KOH or PCA. Samples were then centrifuged at 13,000 x g for 15 min at 4°C and supernatant was collected. ATP was measured using the Tecan Spark 10M plate reader. ATP concentration (nmol/uL) was normalized to protein.

Histology

Kidney sections approximately 5 microns from WT and 5-HT_{1F} receptor mice 24 and 144 hr subjected to sham or I/R surgery were stained with Periodic Acid Schiff staining. Each section was scored based on morphologic changes in a blinded fashion by a renal pathologist (J.M.). The following measures were chosen as an indication of morphologic damage to the kidney: proximal tubule dilation, brush-border damage, proteinaceous casts, interstitial widening and necrosis. 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 ± SEM for all the experiments. Multiple comparisons of normally distributed data were analyzed using one-way analysis of variance (ANOVA) with an appropriate post-hoc test, group means were compared using Holm-Sidak's post-test. Single comparisons were analyzed by Student's *t*-test where appropriate. The criterion for statistical differences was $p \leq 0.05$ for all comparisons.

Table 3.1: Primer sequences used for RT-qPCR.

MOUSE

GENE	PRIMER SEQUENCE	ACCESSION NO.
PGC-1α	Sense 5′-AGGAAATCCGAGCGGAGCTGA-3′ Antisense 5′ GCAAGAAGGCGACACATCGAA-3′	NM_008904.2
NDUFS1	Sense 5′-AGATGATTTGGGAACAACAG-3′ Antisense 5′-TAAGGCTTAGAGGTTAGGGC-3′	NM_001160038.1
ATP Synthase β	Sense 5′-CTATGTGCCTGCTGATGACC-3′ Antisense 5′-GGATAGATGCCCAACTCAGC-3′	NM_016774.3
COXI	Sense 5′-ACCATCATTTCTCCTTCTCC-3′ Antisense 5′-GGTGGGTAGACTGTTCATCC-3′	ENSMUSG00000064351.1
ND1	Sense 5′-TAGAACGCAAAATCTTAGGG-3′ Antisense 5′-TGCTAGTGTGAGTGATAGGG-3′	ENSMUSG00000064341.1
NRF1	Sense 5′-TCGGGCATTTATCCCAGAGATGCT-3′ Antisense 5′-TACGAGATGAGCTATACTGTGTGT-3′	NM_001164226.1
NRF2	Sense 5′-CCTCTGTCACCAGCTCAAGG-3′ Antisense 5′-TTCTGGGCGGCGACTTTATT-3′	NM_010902.4
TFAM	Sense 5′-GCTGATGGGTATGGAGAAG-3′ Antisense 5′-GAGCCGAATCATCCTTTGC-3′	NM_009360.4
DRP1	Sense 5′-GCCTCAGATCGTCGTAGTGG-3′ Antisense 5′-TCTGCTTCAACTCTCCAGCTC-3′	NM_001276340.1
Pink1	Sense 5′-TTGCAATGCCGCTGTGTATG-3′ Antisense 5′-TGGAGGAACCTGCCGAGATA-3′	NM_026880.2
SOD2	Sense 5′-CAAGGGAGATGTTACAACTCAGG-3′ Antisense 5′-CTTAGGGCTCAGGTTTGTCCA-3′	NM_013671.3
UCP2	Sense 5′-GAGATACCAGAGCACTGTCG-3′ Antisense 5′-GTCATCTGTCATGAGGTTGG-3′	NM_011671.5
5-HT_{1B}	Sense 5′-AGACAGGGGTACCTCTCACCAACC-3′ Antisense 5′-ATGAGCGCCAACAAAGCAACCAGC-3′	NM_010482.1
5-HT_{1D}	Sense 5′-AAACCAGTCCCTAGAAGGCCTTCC-3′ Antisense 5′-GCCAGTGTGATGACGGACAGCAC -3′	NM_008309.5
5-HT_{1F}	Sense 5′-GCCGTGATGATGAGTGTGTC-3′ Antisense 5′-ATCATCCGACTCGCTTGTCT-3′	NM_008310.3
5-HT_{2A}	Sense 5′-GCTGAGCCGACAGCTAATGA-3′ Antisense 5′-CATCCGGTCCATCACACACA-3′	NM_172812.2
5-HT_{2B}	Sense 5′-TCTTCAATAAGACATTTCGGGA-3′ Antisense 5′-GAATGGTTGAACTTCGGAGC-3′	NM_008311.2
5-HT_{2C}	Sense 5′-AGATATTTGTGCCCCGTCTG-3′ Antisense 5′-GCCTTAGTCCGCGAATTGAA-3′	NM_008312.4
β-Actin	Sense 5′-GGGATGTTTGCTCCAACCAA-3′ Antisense 5′-GCGCTTTTGACTCAGGATTTAA-3′	NM_007393.5

HUMAN

GENE	PRIMER SEQUENCE	ACCESSION NO.
5-HT_{1B}	Sense 5′-ACGCCGTGGAGTACTCAGCTAAAAG-3′ Antisense 5′-GGAGTAGACCGTGTAGAGGATGTGG-3′	NM_000863.2
5-HT_{1D}	Sense 5′-TGCCGTGGTCCTTTCCGTC-3′ Antisense 5′-GGTGATGGTATAGGCGATGCTG-3′	NM_000864.4
5-HT_{1F}	Sense 5′-GCTATAGCTTTGGATCGGTATCGAG-3′ Antisense 5′-CAATCCTACTTGCTTGTCTCTTGTG-3′	NM_001322209.1
5-HT_{2A}	Sense 5′-TCGCCATCCAGAATCCCATCCACC-3′ Antisense 5′-GATGGACTGCATAGTCCTCCTGCC-3′	NM_001165947.2
5-HT_{2B}	Sense 5′-ACGCCTAACATGGTTGACTGTGTC-3′ Antisense 5′-TGAGGCTCTCTGTCCGTTGGAA-3′	NM_000867.4
5-HT_{2C}	Sense 5′-TAACACTGAAGCAATCATGG-3′ Antisense 5′-GACTGTGCTGTTCTTTCTCACAC-3′	NM_001256761.2
β-Actin	Sense 5′-CTGGAACGGTGAAGGTGACA-3′ Antisense 5′-GAAGGGACTTCCTGTAACAATGCA-3′	NM_001101.3

RESULTS

Renal and cardiac 5-HT_{1F} receptor gene absence in 5-HT_{1F} receptor knockout mice

Genomic DNA was isolated from the tails of pups derived from a cross of two heterozygous mice followed by agarose gel electrophoresis to verify loss of the 5-HT_{1F} receptor gene. WT mice only expressed the wild type 5-HT_{1F} receptor PCR product (163 bp), KO mice only expressed mutant PCR product specific to the sequence targeted to disrupt the 5-HT_{1F} receptor gene (509 bp), and HET mice expressed both the wild type and mutant PCR product (Fig. 3.1A). In addition, HET x HET matings yielded Mendelian ratios of 1:2:1 of WT, HET, and KO. KO x KO matings also yielded viable mice. To verify that the presence of the mutant gene caused a decrease in the 5-HT_{1F} receptor, we measured 5-HT_{1F} receptor mRNA. Renal and cardiac 5-HT_{1F} mRNA was decreased 50% in HET mice and absent in KO mice (Fig. 3.1B, C). These findings confirm the successful breeding of 5-HT_{1F} receptor KO mice.

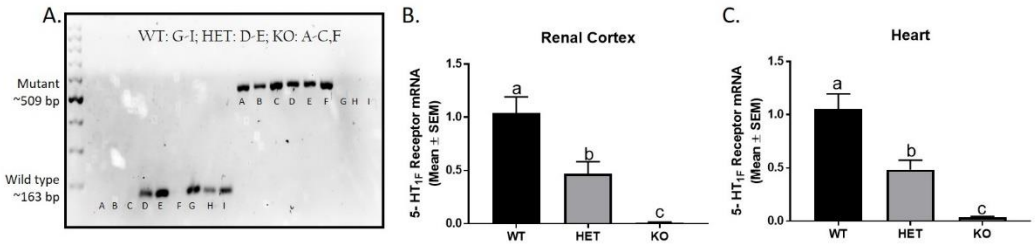
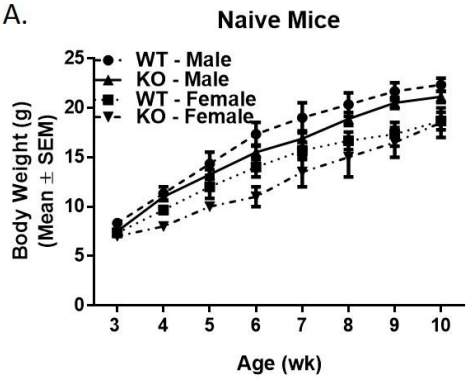


Figure 3.1: Renal and cardiac 5-HT_{1F} receptor gene absent in 5-HT_{1F} receptor knockout mice. Genotyping of 5-HT_{1F} receptor using PCR and subjected to 2.5% agarose gel electrophoresis. Wild-type 5-HT_{1F} receptor PCR product is 163 bp and mutated 5-HT_{1F} receptor PCR product is 509 bp. 100bp DNA ladder is shown in the far left lane. Mice expressing only the wild- type 5-HT_{1F} receptor PCR product is WT, mice expression only the mutated 5-HT_{1F} receptor PCR product is KO, and mice expressing both the wild- type and mutated 5-HT_{1F} receptor PCR product is HET (A). Expression of renal 5-HT_{1F} receptor was measured at the mRNA level in WT, HET, and KO mice (B). Expression of cardiac 5-HT_{1F} receptor was measured at the mRNA level in WT, HET, and KO mice (C). Data are reported as mean ± SEM, n = 6. *$p<0.05$ versus WT controls

The lack of 5-HT_{1F} receptor does not affect body weight, blood counts or serum chemistries

We compared WT to KO mice with respect to weight gain, serum chemistry and complete blood count at 10 weeks of age. Male and female WT and KO mice were weighed weekly for 3-10 weeks. There were no differences in weight gain with age between WT and KO mice (male or female) (Fig. 3.2A). There were also no differences in white blood cells, red blood cells or in any serum chemistries between WT and KO mice (Fig. 3.2B). Thus, the loss of the 5-HT_{1F} receptor did not alter body weight, electrolyte, metabolic, or blood cell composition at early ages.

A.



B.

PARAMETER (UNITS)	WT (MEAN ± SEM)	KO (MEAN ± SEM)
WBC (K/µL)	9.5 ± 0.6	11.3 ± 1.3
Neutrophils (K/µL)	2.2 ± 0.2	2.9 ± 0.3
Lymphocytes (K/µL)	6.6 ± 0.3	7.6 ± 0.9
RBC (M/µL)	10.5 ± 0.3	10.1 ± 0.3
Albumin (g/dL)	2.9 ± 0.7	3.4 ± 0.0
Total Protein (g/dL)	5.5 ± 0.2	5.6 ± 0.1
Calcium (mg/dL)	11.4 ± 0.2	11.3 ± 0.2
Sodium (mmol/L)	152 ± 2	153 ± 1
Potassium (mmol/L)	10.5 ± 0.8	10.0 ± 0.7
Chloride (mmol/L)	115 ± 1	115 ± 1
Glucose (mg/dL)	269 ± 13	251 ± 12
Creatinine (mg/dL)	0.3 ± 0.2	0.2 ± 0.1

Figure 3.2: The lack of 5-HT$_{1F}$ receptor does not affect body weight, blood counts or serum chemistries. Body weight of 5-HT$_{1F}$ receptor WT and KO male and female mice from 3 to 10 weeks of age (A). Complete blood count and serum chemistries of 5-HT$_{1F}$ WT and KO mice at 10 weeks of age (B).Data are reported as mean ± SEM, n = 5-6.

The lack of the 5-HT_{1F} receptor does not induce renal injury

We evaluated BUN in WT and KO mice. There was no difference in BUN levels at 10 or 26 weeks of age (10 week WT: 26 ± 3 mg/dL, 10 week KO: 25 ± 2 mg/dL; 26 week WT: 21 ± 1 mg/dL, 26 week KO: 30 ± 1 mg/dL). To further assess if the absence of the 5-HT_{1F} receptor induced renal tubular injury, kidney injury molecule-1 (KIM-1) and neutrophil gelatinase-associated lipocalin (NGAL) were evaluated in the renal cortex of WT and KO mice. At 10 and 26 weeks, KIM-1 was not detected and NGAL was unchanged in KO mice compared to WT mice (Fig. 3.3A, B). Taken together, these data suggest that the KO of the 5-HT_{1F} receptor alone does not cause renal injury at ages up to 26 weeks.

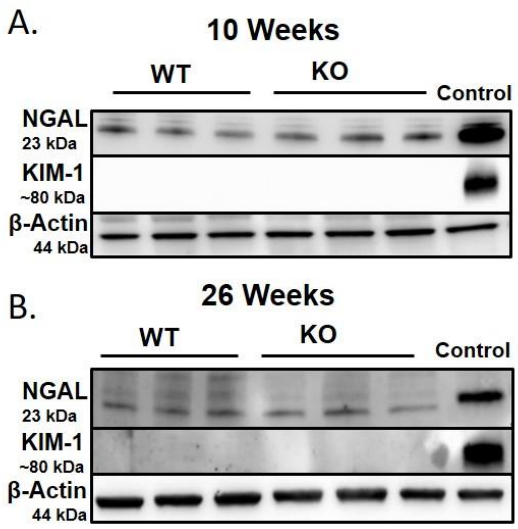


Figure 3.3: The lack of the 5-HT$_{1F}$ receptor does not induce renal injury. Immunoblot analysis of renal cortical KIM-1 and NGAL expression was used to assess tubular injury in 10 week (A) and 26 week (B) old male 5-HT$_{1F}$ receptor WT and KO mice.

Altered renal mitochondrial homeostasis in young and aged 5-HT_{1F} KO mice

Our laboratory demonstrated that stimulation of the 5-HT_{1F} receptor results in increased MB as measured by increased expression of mitochondrial proteins and mtDNA copy number (5). Furthermore, knockdown of the 5-HT_{1F} receptor in renal proximal tubular cells resulted in a decrease in mitochondrial proteins (5). Thus, we hypothesized that the absence of the 5-HT_{1F} receptor would lead to a reduction in mitochondrial markers in the renal cortex. We assessed mRNA expression of MB proteins and components of the ETC, including PGC-1α, nuclear respiratory factor-1 (Nrf-1), nuclear respiratory factor-2 (Nrf-2), mitochondrial transcription factor A (TFAM), and nuclear-encoded NADH dehydrogenase (ubiquinone) FE-S protein 1 (NDUFS1), ATP synthase β (ATPSβ), and mitochondrial-encoded cytochrome c oxidase subunit 1 (COX1) and NADH dehydrogenase 1 (ND1). Transcript levels of Nrf-2, TFAM, NDUFS1, ATPSβ, and COX1 were increased (1.4-,1.4-, 1.7-, 1.3-, and 1.6-fold, respectively) at 10 weeks and these increases corresponded with elevated mtDNA copy number (Fig. 3.4A). At 26 weeks, mRNA expression of PGC-1α and COX1 were elevated 1.5- and 1.8-fold, respectively, compared to WT levels and these changes were also accompanied by a 1.7-fold increase in mtDNA copy number, whereas NDUFS1, ATPSβ, and ND1 transcript levels returned to WT levels (Fig. 3.4C). These results reveal the loss of the 5-HT_{1F} receptor leads to increased MB genes in the kidney.

Mitochondrial fission and mitophagy are critical processes in maintaining mitochondrial homeostasis.(14, 15) As such, we measured markers of mitochondrial fission, dynamin-related protein 1 (Drp1) and mitophagy, mitochondrial PTEN-induced putative kinase 1 (PINK1). At 10 weeks, Drp1 and PINK1 were elevated 1.7- and 1.6-fold, respectively (Fig. 3.4B). In contrast, at 26 weeks, PINK1 was decreased 40%, yet Drp1 was maintained at a 1.6-fold increase compared to WT mice (Fig. 3.4D). These results reveal

that mitochondrial fission and mitophagy may be increased in response to the loss of the 5-HT_{1F} receptor.

Finally, we assessed superoxide dismutase 2 (SOD2) and uncoupled protein 2 (UCP2), markers of oxidative stress and mitochondrial uncoupling (16, 17). UCP2 mRNA, but not SOD2 was elevated 1.4-fold at 10 weeks; however, both UCP2 and SOD2 transcript levels were unchanged compared to WT mice at 26 weeks (Fig. 3.4B,D). These findings reveal that renal UCP2 is transiently altered in 5-HT_{1F} receptor KO mice.

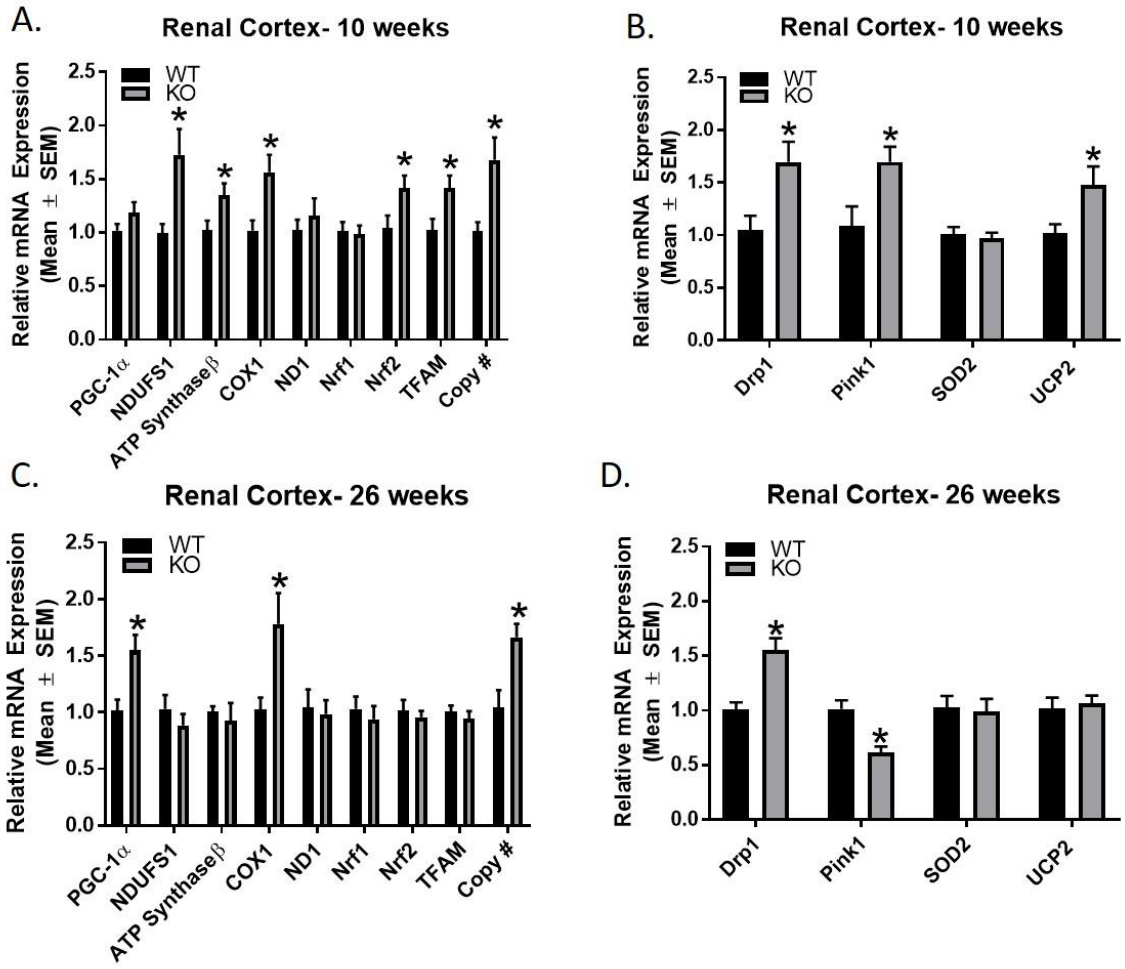


Figure 3.4: Altered renal mitochondrial homeostasis in young and aged 5- HT_{1F} KO mice. Total RNA was harvested from renal cortical tissue of 5-HT_{1F} receptor WT and KO mice. Gene expression of key regulators of MB, fission, oxidative stress were measured on the mRNA level at 10 weeks (A, C) and 26 weeks (B,D) of age.. Relative mitochondrial DNA content in the renal cortex was determined by qRT-PCR analysis at 10 weeks (A) and 26 weeks (B) of age. Data are reported as mean ± SEM, n = 6. *p<0.05 versus WT controls.

Altered cardiac mitochondria homeostasis in young and aged 5-HT$_{1F}$ KO mice

Because stimulation of the 5-HT$_{1F}$ receptor induced cardiac MB in mice, we hypothesized that the absence of the 5-HT$_{1F}$ receptor would also lead to disruption of mitochondrial homeostasis in the heart. In contrast to the changes observed in renal cortical tissue, cardiac mRNA of PGC-1α, Nrf-1, Nrf-2, TFAM, ATPSβ, COX1, ND1, and mtDNA copy number were decreased (40, 35, 40, 35, 40, 55, 40, and 30%, respectively) at 10 weeks of age (Fig. 3.5A). All genes returned to WT levels except PGC-1α and TFAM, which were both increased 1.8-fold compared to WT levels at 26 weeks of age (Fig. 3.5C). These results reveal that the loss of the 5-HT$_{1F}$ receptor leads to decreased MB genes at 10 weeks of age and mostly return to WT levels at 26 weeks of age in the heart.

Cardiac gene expression of Drp1, PINK1 and SOD2 were reduced (45, 40, 35%, respectively) in KO mice at 10 weeks of age (Fig. 3.5B). However, PINK1, and UCP2 transcript levels were elevated 1.6- and 1.5-fold, respectively, in KO mice at 26 weeks of age (Fig. 3.5D).

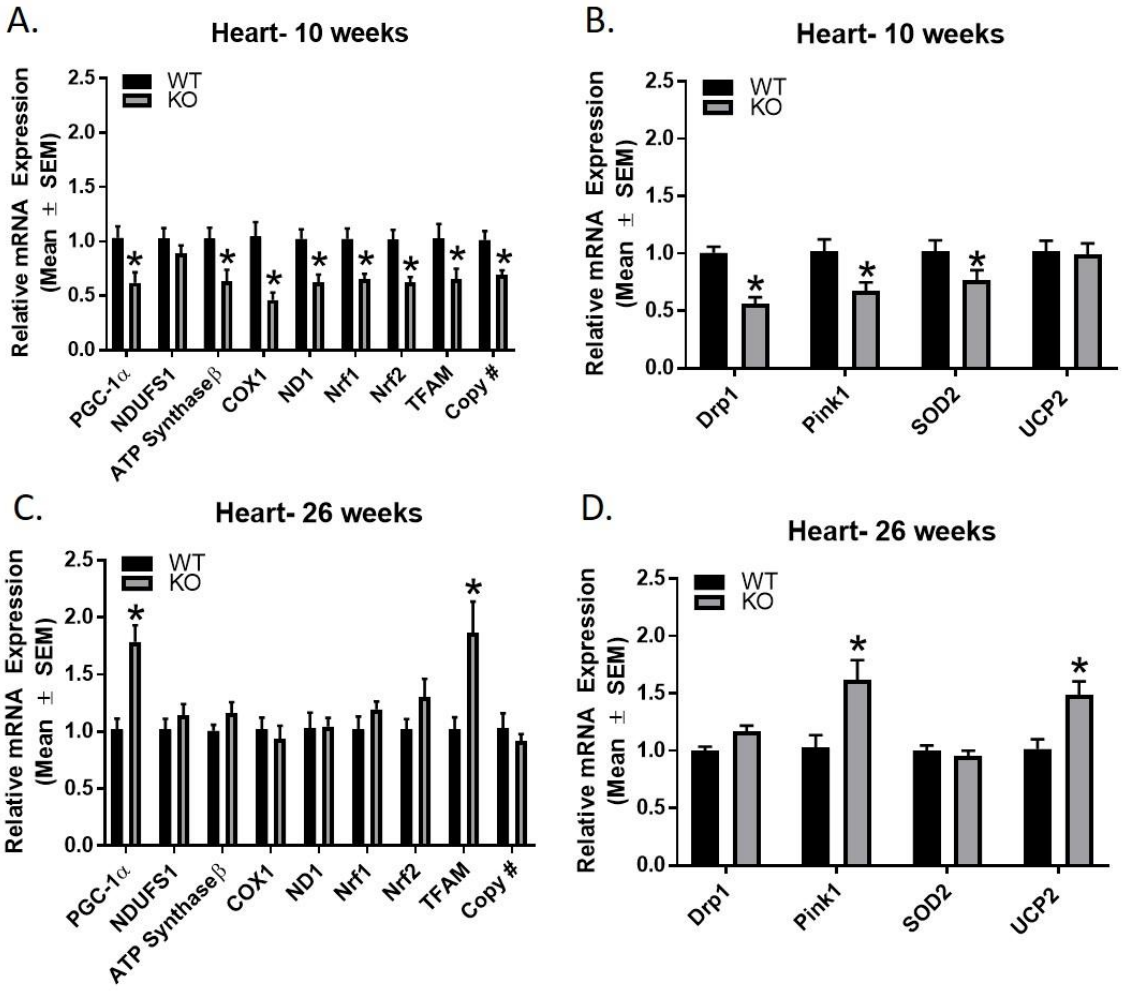


Figure 3.5: Altered cardiac mitochondrial homeostasis in young and aged 5- HT_{1F} KO mice. Total RNA was harvested from left ventricle cardiac tissue of 5-HT_{1F} receptor WT and KO mice. Gene expression of key regulators of MB, fission, oxidative stress were measured on the mRNA level at 10 weeks (A, C) and 26 weeks (B,D) of age.. Relative mitochondrial DNA content in left ventricle was determined by qRT-PCR analysis at 10 weeks (A) and 26 weeks (B) of age. Data are reported as mean ± SEM, n = 6. *p<0.05 versus WT controls.

Altered renal 5-HT receptor gene expression in 5-HT_{1F} KO mice

We explored the possibility that the lack of the 5-HT_{1F} receptor results in altered gene expression of other 5-HT receptors. Absence of the 5-HT_{1F} receptor decreased renal mRNA of 5-HT_{1B}, 5-HT_{1D}, and 5-HT_{2A}, receptors (~30, 35, 45%) at 10 weeks, while 5-HT_{2B} receptor mRNA increased ~2.6-fold and 5-HT_{2C} receptor mRNA did not change compared to WT (Fig. 3.6A). By 26 weeks of age the transcripts of 5-HT_{1D}, 5-HT_{2A} and 5-HT_{2C} receptors were elevated by ~2.3-, 1.6-, and 1.7-fold, respectively, whereas 5-HT_{1B} and 5-HT_{2B} receptor mRNA returned to WT levels (Fig. 3.6B). Thus, altered renal 5-HT receptor gene expression may be a compensatory mechanism in response to the absence of the 5-HT_{1F} gene.

We also measured cardiac 5-HT receptor gene expression in response to 5-HT_{1F} receptor absence. Cardiac 5-HT_{1D} and 5-HT_{2B} receptor mRNA expression were both decreased by ~40% at 10 weeks, while 5-HT_{2A} receptor mRNA increased 1.6-fold compared to WT levels (Fig. 3.6C). At 26 weeks, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{2A}, and 5-HT_{2C} receptor mRNA were elevated ~1.2-, 1.6-, 2.7-, and 1.7- fold, respectively, in KO mice (Fig. 3.6D). These results reveal that compensatory mechanisms may be tissue-specific, producing differences in mitochondrial gene expression in the kidney and heart.

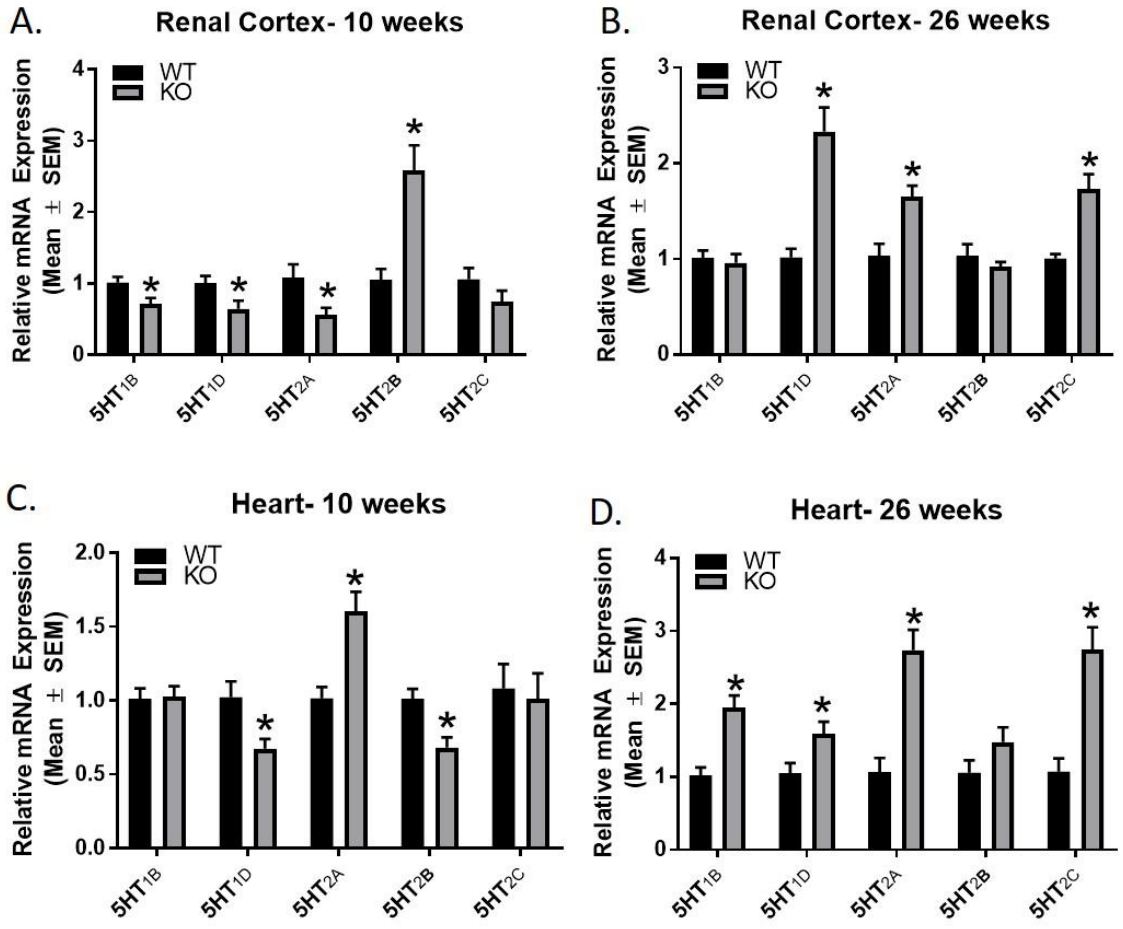


Figure 3.6: Altered renal and cardiac 5-HT receptor gene expression in KO mice. Total RNA was harvested from renal cortical and left ventricle cardiac tissue of 5-HT_{1F} receptor WT and KO mice. Expression of 5-HT_{1B}, 5-HT_{1D}, 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C} were measured on the mRNA level at 10 weeks (A, C) and 26 weeks (B,D) of age. Data are reported as mean ± SEM, n = 6. *p<0.05 versus WT controls.

The absence of the 5-HT_{1F} receptor potentiates I/R-induced AKI at 24 hr

While the absence of the 5-HT_{1F} receptor did not cause renal injury, it is possible that under stress the absence of the 5-HT_{1F} receptor could potentiate renal injury. To test this hypothesis, we subjected 10-week-old WT and KO mice to renal I/R injury. Renal function was assessed 24 hr after I/R-induced AKI and an increase in serum creatinine and BUN in WT and KO AKI mice was observed compared to sham controls. However, there was no statistical difference in serum creatinine or BUN between genotypes (Fig. 3.7A,B). To assess renal cortical injury in this model, we measured two markers of renal injury, KIM-1 and NGAL protein in the renal cortex at 24 hr. KIM-1 and NGAL increased 3- and 2-fold, respectively, in 5-HT_{1F} receptor KO mice subjected to I/R injury compared to WT mice subjected to IR injury (Fig. 3.7C-E). It is also important to note 5-HT_{1F} receptor mRNA decreased by ~50% in WT mice subjected to I/R (Fig. 3.7F).

Renal histopathology was assessed using periodic acid-Schiff (PAS) staining. Kidneys from both WT and 5-HT_{1F} receptor KO mice subjected to I/R injury exhibited proximal tubule necrosis and brush border damage. However, the absence of the 5-HT_{1F} receptor did not potentiate tubular necrosis or brush border damage at 24 hr (Fig. 3.8). Despite the renal histology findings, these data indicate that I/R-induced AKI results in depletion of the 5-HT_{1F} receptor mRNA and that the loss of this receptor potentiates renal injury and suggests that the 5-HT_{1F} receptor is renal protective under stress.

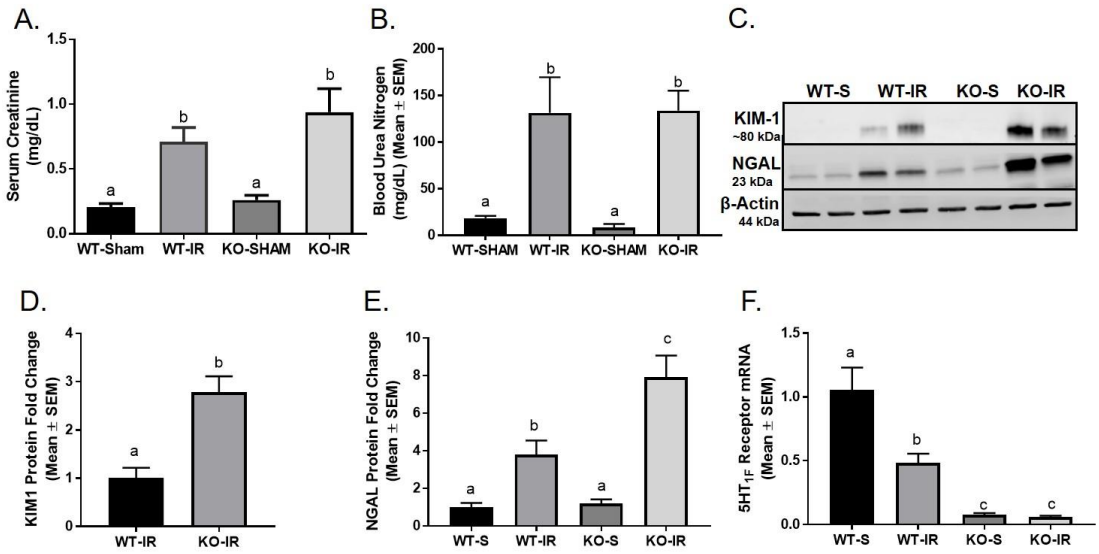


Figure 3.7: The absence of the 5-HT$_{1F}$ receptor potentiates I/R- induced AKI at 24 hr. Serum creatinine (A) and BUN (B) was assessed 24 hr after renal I/R injury in WT and 5-HT$_{1F}$ receptor KO mice. Immunoblot analysis of renal cortical KIM-1 and NGAL expression was used to asses tubular injury in WT and 5-HT$_{1F}$ receptor KO mice following I/R- induced AKI (C). Densitometry analysis of KIM-1 (D) and NGAL (E) following I/R injury. Total RNA was harvested from renal cortical of WT and 5-HT$_{1F}$ receptor KO mice following I/R- induced AKI. Expression of 5-HT$_{1F}$ receptor mRNA was measured (E).Data are reported as mean ± SEM, n = 4-6. Different superscripts indicate statistically significant differences ($p<0.05$).

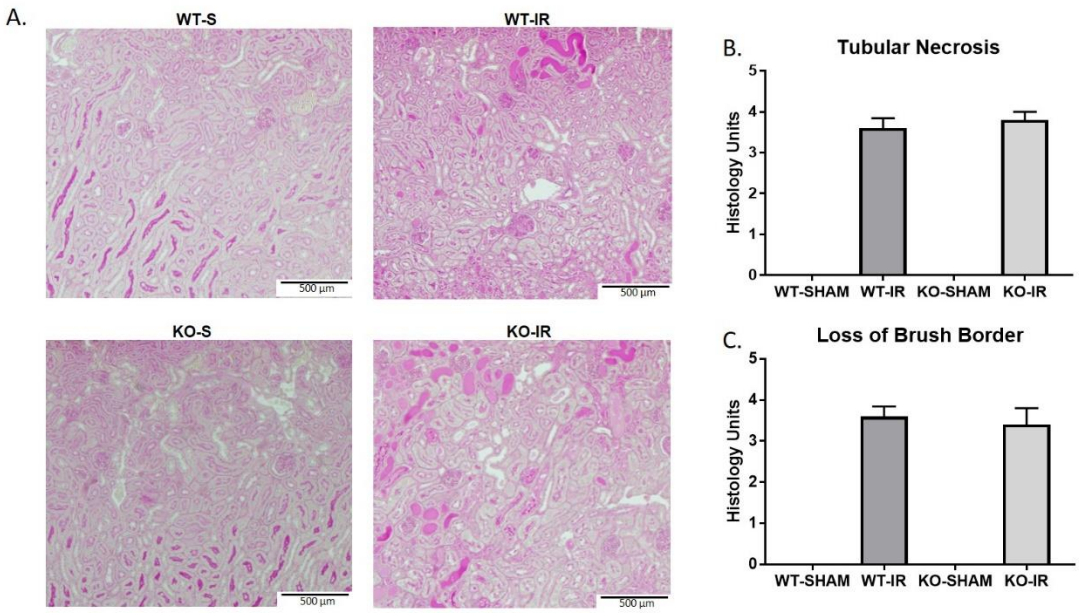


Figure 3.8: Absence of the 5-HT$_{1F}$ receptor has no effect on renal cortical histology at 24 hr. Renal cortical sections of WT and 5-HT1F receptor KO mice subjected to sham or I/R-induced AKI were stained with Periodic acid-Schiff (PAS). PAS stain of representative slides of renal cortical tissues at 24 hr (magnification, x100x) (A). Tubular necrosis scoring (B). Loss of brush border scoring(C).Data are reported as mean ± SEM, n = 5-6.

The absence of the 5-HT_{1F} receptor reduces the recovery of renal function

A previous study demonstrated that the stimulation of MB though the activation of the 5-HT_{1F} receptor accelerates the recovery of renal function and tubular injury after AKI. Thus, we postulated that the absence of the 5-HT_{1F} receptor would reduce recovery of renal function following injury (5). Renal function and tubular injury were assessed at 24 hr and 144 hr following I/R-induced AKI in WT and KO mice. All I/R mice had equal initial injury with an overall average serum creatinine of 0.7 ± 0.2 mg/dL and BUN of 67 ± 23 mg/dL. However, 5-HT_{1F} receptor KO and WT mice failed to recover renal function as demonstrated by persistent overall elevated serum creatinine and BUN levels of 0.5 ± 0.2 mg/dL and 52 ± 23 mg/dL, respectively at 144 hr (Fig.3.9 A, C). It is noteworthy to mention that tubular necrosis and brush border damage remained elevated at 144 hr following I/R injury, however, renal histology scoring revealed that all mice exhibited the same degree of tubular necrosis and loss of brush border (Fig.3.10).

To further explore renal recovery in 5-HT_{1F} receptor KO mice, renal function improvement was calculated as change in serum creatinine and BUN (delta serum creatinine and BUN) at 144 hr compared to 24 hr following AKI in each mouse. Using this analysis serum creatinine decreased ~0.25 mg/dL in AKI WT mice compared to no decrease in serum creatinine in AKI KO mice (Fig. 3.9 B). Similar results were observed in BUN which decreased ~35 mg/dL in AKI WT mice compared to no decrease in AKI KO mice (Fig. 3.9D).

To assess renal tubular injury, renal cortical KIM-1 and NGAL levels were measured by immunoblot analysis. KIM-1 and NGAL were upregulated by 2- and 3- fold, respectively, in 5-HT_{1F} receptor KO mice subjected to I/R injury compared to WT mice (Fig.3.9E-G). Taken together, these findings provide strong evidence that the loss of the 5-HT_{1F} receptor potentiates AKI and is key to facilitate renal recovery.

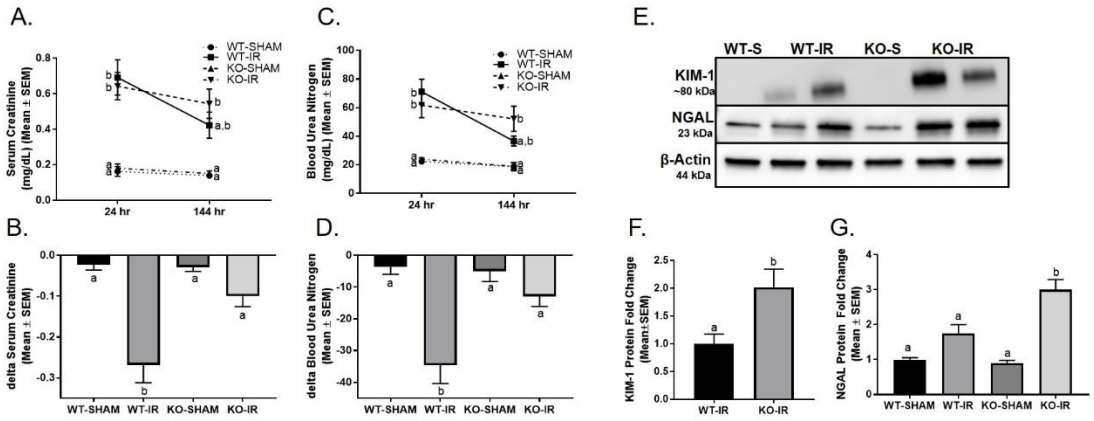


Figure 3.9. The absence of the 5-HT$_{1F}$ receptor reduces the recovery of renal function. Serum creatinine(A), delta serum creatinine (B), BUN (C) and delta BUN (D) were assessed 144 hr after renal I/R injury in WT and 5-HT$_{1F}$ receptor KO mice. Immunoblot analysis of renal cortical KIM-1 and NGAL expression was used to asses tubular injury in WT and 5-HT$_{1F}$ receptor KO mice following I/R- induced AKI (E). Densitometry analysis of KIM-1 (F) and NGAL (G) following I/R injury. Data are reported as mean ± SEM, n = 4-6. Different superscripts indicate statistically significant differences ($p<0.05$).

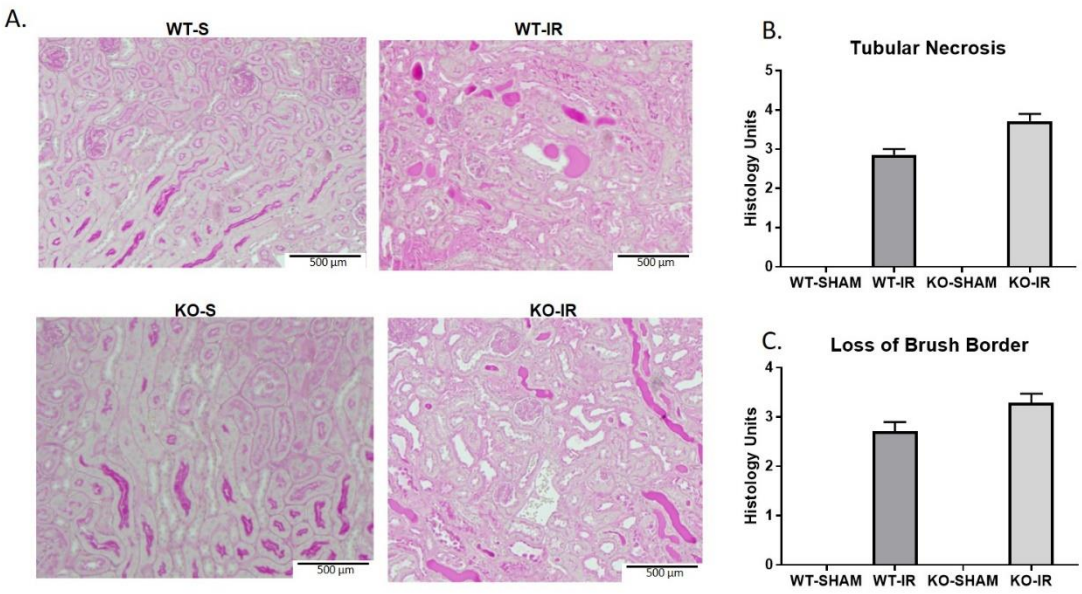


Figure 3.10: Absence of the 5-HT$_{1F}$ receptor has no effect on renal cortical histology at 144 hr. Renal cortical sections of WT and 5-HT1F receptor KO mice subjected to sham or I/R-induced AKI were stained with Periodic acid-Schiff (PAS). PAS stain of representative slides of renal cortical tissues at 144 hr (magnification, x100x) (A). Tubular necrosis scoring (B). Loss of brush border scoring(C).Data are reported as mean ± SEM, n = 5-6.

The absence of the 5-HT_{1F} receptor suppresses MB during I/R- induced AKI

To determine whether greater tubular injury in the 5-HT_{1F} receptor KO mice was associated with suppressed MB, we assessed renal cortical PGC-1α, NDUFS1, COX1 and TFAM protein at 24 and 144 hr in mice subjected to I/R. PGC-1α and TFAM protein were reduced ~50-60% in KO mice after I/R-induced AKI compared to WT and KO sham mice at 24 hr (Fig. 3.11A, C, F). No difference in PGC-1α and TFAM protein was detected in I/R injured WT mice (Fig. 3.11A, C, F). We assessed the same endpoints at 144 hr following I/R injury. Renal cortical PGC-1α, NDUFS1 and COX1 protein were reduced by ~50, 75, 50%, respectively, in 5-HT_{1F} receptor KO mice compared to sham animals of both genotypes at 144 hr (Fig.3.11 B, G, H). It is important to note that NDUFS1 protein was reduced at 144 hr in WT mice following injury and to a further extent in 5-HT_{1F} receptor KO mice subjected with I/R (Fig. 3.11 B,H). Similar findings were observed following mRNA analysis of these endpoints (Fig. 3.12). These findings suggest that MB is persistently suppressed following AKI and to a greater magnitude in the absence of the 5-HT_{1F} receptor.

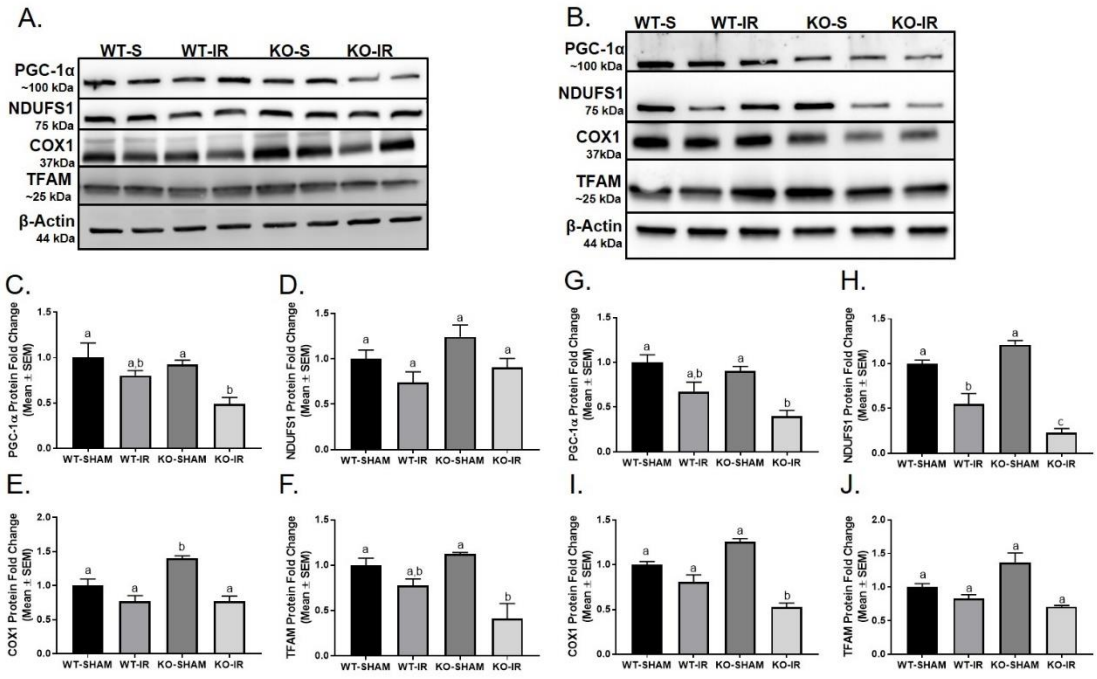


Figure 3.11: The absence of the 5-HT$_{1F}$ receptor suppresses MB during I/R-induced AKI. Total protein was harvested from the renal cortex of WT and 5-HT$_{1F}$ receptor KO mice following I/R- induced AKI. Immunoblot analysis of renal cortical PGC-1α , NDUFS1, COX1 and TFAM protein expression was used to assess MB in WT and 5-HT$_{1F}$ receptor KO mice following I/R- induced AKI at 24 hr (A) and 144 hr (B). Densitometry analysis of 24 hr PGC-1α (C), NDUFS1 (D), COX1 (E) and TFAM (F) and 144 hr PGC-1α (G), NDUFS1 (H), COX1 (I) and TFAM (J) following I/R injury. Data are reported as mean ± SEM, n = 4-6. Different superscripts indicate statistically significant differences ($p<0.05$).

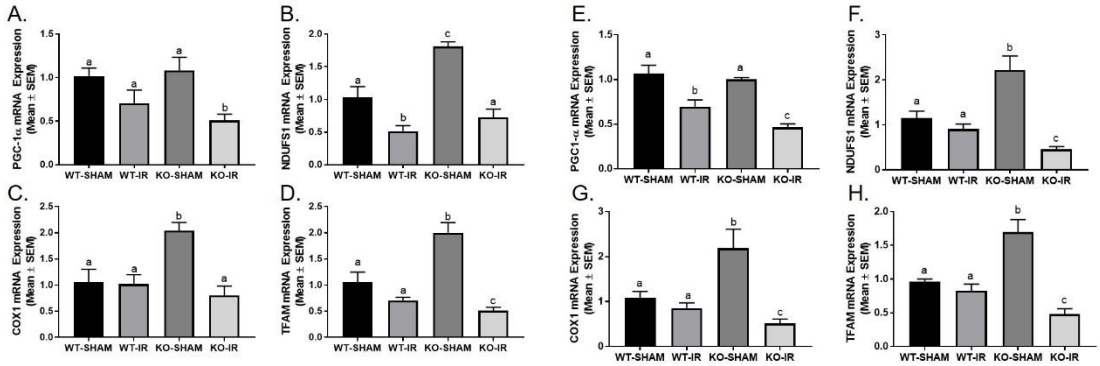


Figure 3.12: The absence of the 5-HT$_{1F}$ receptor disrupts gene expression of mitochondrial homeostasis following I/R-induced AKI. Total RNA was extracted from renal cortical tissue. Gene expression of 24 hr PGC-1α (A), NDUFS1 (B), COX1 (C), TFAM (D) and 144 hr PGC-1α (E), NDUFS1 (F), COX1 (G), TFAM (H) were measured to assess mitochondrial homeostasis following I/R- induced renal injury. Data are reported as mean ± SEM, n = 4-6. Different superscripts indicate statistically significant differences ($p<0.05$).

5-HT$_{1F}$ receptor KO mice exhibit persistent decrease in ATP levels following I/R-induced renal injury

To assess whether the absence of the 5-HT$_{1F}$ receptor alters mitochondrial function following I/R-induced AKI, cortical ATP levels were measured. ATP was reduced by ~50% following I/R injury in both genotypes at 24 hr (Fig. 3.13A). However, WT mice exhibited partial recovery in ATP content by 144 hr, which was absent in the 5-HT$_{1F}$ receptor KO mice (Fig. 3.13B).

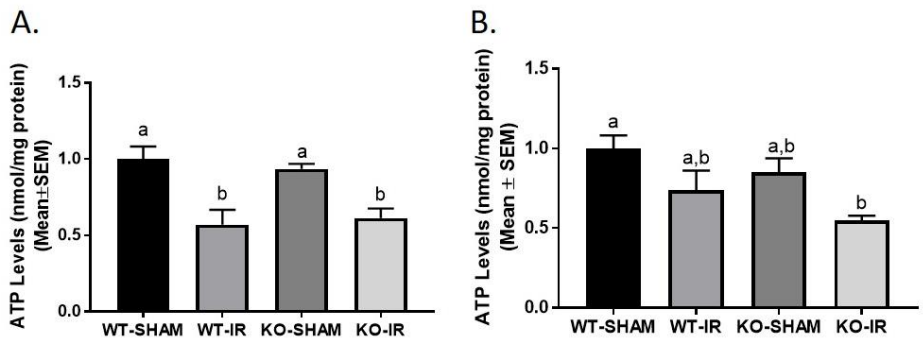


Figure 3.13: 5-HT_{1F} receptor KO mice exhibit persistent decrease in ATP levels following I/R- induced renal injury. Renal cortical ATP concentration (nmol/uL) was measured in WT and 5-HT_{1F} receptor KO mice 24 hr (A) and 144 hr (B) following renal I/R injury and then normalized to wet weight of protein (mg). Data are reported as mean ± SEM, n = 4-6. Different superscripts indicate statistically significant differences ($p<0.05$).

The absence of the 5-HT_{1F} receptor increases oxidative stress at 144 hr following I/R- induced AKI

Previous studies reported that renal I/R results in oxidative stress. (18) Thus, we measured 4 hydroxy-2-nonenal (4-HNE) protein modifications as a marker of oxidative stress in renal cortical proteins at 24 and 144 hr in WT and 5-HT_{1F} receptor KO mice subjected to I/R. At 24 hr no differences were detected in any group (Fig. 3.14). In the absence of the 5-HT_{1F} receptor 4-HNE protein modifications increased by 1.5- fold compared to WT mice following I/R injury, suggesting increased oxidative stress in KO mice.

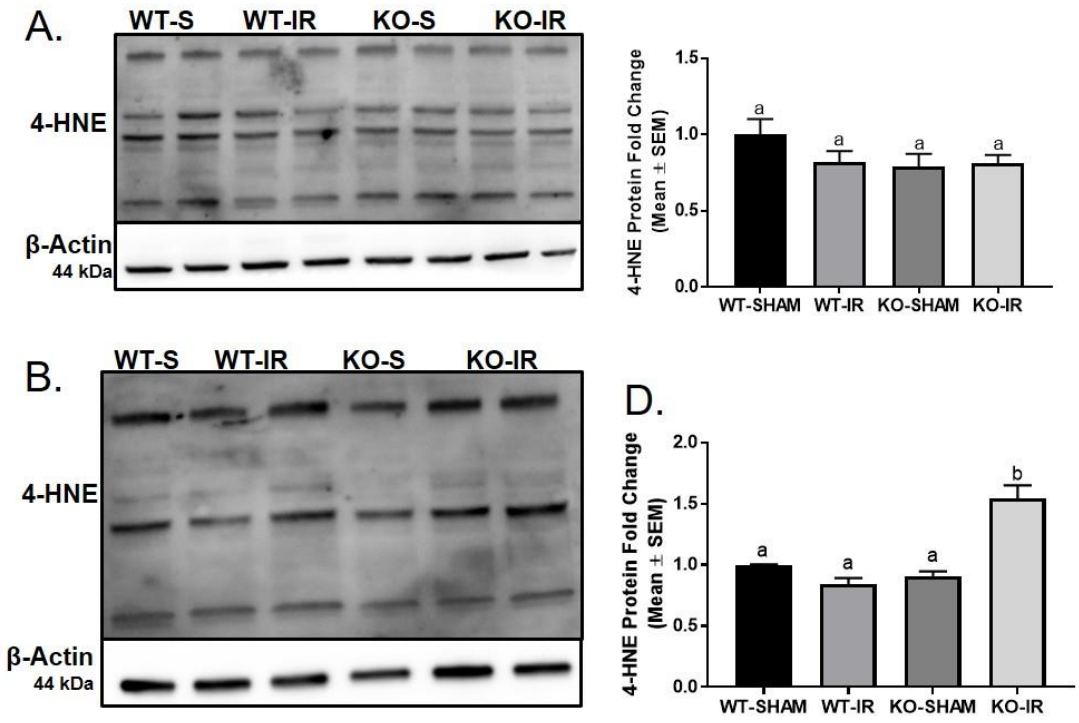


Figure 3.14: The absence of the 5-HT$_{1F}$ receptor induces oxidative stress at 144 hr following I/R- induced AKI. Total protein was harvested from the renal cortex of WT and 5-HT$_{1F}$ receptor KO mice following I/R- induced AKI. Immunoblot analysis of renal cortical 4-HNE protein expression was used to assess oxidative stress in WT and 5-HT$_{1F}$ receptor KO mice following I/R- induced AKI at 24 hr (A) and 144 hr (B). Densitometry analysis of 24 hr 4-HNE (C) and 144 hr 4-HNE (D) following I/R injury. Data are reported as mean ± SEM, n = 4-6. Different superscripts indicate statistically significant differences ($p<0.05$) versus WT controls

5-HT$_1$ and 5-HT$_2$ receptors are expressed in human kidney and heart

To determine the translatability of this model to human disease, we measured gene expression of 5-HT$_1$ and 5-HT$_2$ receptors in renal and cardiac human samples. 5-HT$_{1B}$, 5-HT$_{1D}$, 5-HT$_{1F}$, 5-HT$_{2A}$, 5-HT$_{2B}$, and 5-HT$_{2C}$ receptor gene expression were all detected in both renal and cardiac human samples (Fig. 3.15A, B).

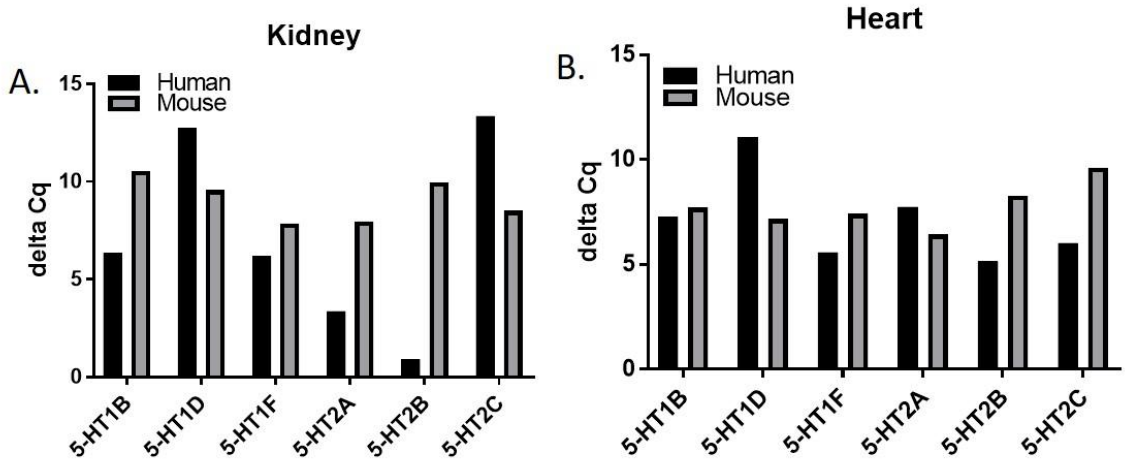


Figure 3.15: 5-HT$_1$ and 5-HT$_2$ Receptors are Expressed in Human Kidney and Heart. Total RNA was extracted from human and mouse renal and cardiac tissue. Expression of renal and left ventricle 5-HT$_{1B}$, 5-HT$_{1D}$, 5-HT$_{1F}$, 5-HT$_{2A}$, 5-HT$_{2B}$ and 5-HT$_{2C}$ receptors were measured on the mRNA level in human and mouse (A, B). Data reported as delta Cq values.

DISCUSSION

Mice homozygous for the mutated 5-HT_{1F} receptor gene were viable and physically similar to WT mice. While the 5-HT_{1F} receptor KO mice did not have gross morphological alterations, nor changes in blood counts and serum chemistries, our data provide clear evidence that mitochondrial homeostasis in renal and cardiac tissue is disrupted. Assimilating various mitochondrial markers revealed that the loss of the 5-HT_{1F} receptor gene resulted in MB and increased mtDNA copy number in the kidney. Associated with increased markers of MB was an increase in the mitochondrial fission protein Drp1 and mitophagy marker, PINK1. One explanation for these results is that the loss of the 5-HT_{1F} receptor and its associated basal control over MB results in a compensatory response to increase MB. Consequently, we hypothesize that mitochondrial fission and mitophagy are increased to maintain a given level of healthy mitochondria.

It is also possible that another 5-HT receptor may compensate for the loss of the 5-HT_{1F} receptor. This hypothesis is supported by a body of evidence indicating functional cross talk as well as direct interaction between different 5-HT receptors. For example, the functional cross talk between 5-HT_{2A} and 5-HT_{2C} receptors, between 5-HT_{1B} and 5-HT_{2C} receptors and the 5-HT_{1A} and 5-HT_{7} receptors (19-21). In this study, examination of other 5-HT receptors revealed altered gene expression in response to the absence of the 5-HT_{1F} receptor. Only the 5-HT_{2B} receptor was upregulated at 10 weeks in the kidney of the 5-HT_{1F} KO mouse. Interestingly, the 5-HT_{2B} receptor is associated with mitochondrial function in the heart (22, 23). At 26 weeks of age the 5-$HT_{1D,\ 2A,\ 2C}$ receptors were upregulated in the 5-HT_{1F} receptor KO mouse. While the 5-HT_{1D} receptor has not been linked to mitochondria, 5-$HT_{2A,2C}$ receptors have been linked to mitochondrial function in the kidney (24). In fact, 5-HT_{2A} receptor agonists induce MB in renal proximal tubular cells (24). Other possibilities exist and future studies are needed

to identify the mechanism by which MB is induced in the absence of the 5-HT_{1F} receptor and the potential role of the other 5-HT receptors.

In marked contrast to the kidney, cardiac mtDNA content and markers involved in MB, fission, and autophagy were diminished in young 5-HT_{1F} receptor KO mice. These findings suggest that compensatory pathways in the young 5-HT_{1F} receptor KO heart are different and not sufficient to maintain mitochondrial homeostasis, leading to suppressed MB, fission and mitophagy. Down regulation of genes involved in fission and autophagy may indicate a mitochondrial preservation pathway that occurs in the absence of a MB signal. In the aged 5-HT_{1F} receptor KO heart, an unknown compensatory pathway develops, as PGC-1α and TFAM mRNA are increased, while other mitochondrial markers have returned to levels found in WT mice.

Examination of other 5-HT receptors in the heart of 5-HT_{1F} receptor KO mice revealed a different pattern of induction compared to the kidney. 5-HT_{2B} receptor gene expression was downregulated and 5-HT_{2A} receptor gene expression was upregulated in the heart at 10 weeks. Absence of the 5-HT_{2B} receptor has been linked to marked reductions in ventricular mitochondrial enzyme activities, mitochondrial number and increased myocardial cell death (22). It is possible that the cardiac 5-HT_{2B} receptor plays a role in modulating MB in the absence of the 5-HT_{1F} receptor. Furthermore, it is also possible that the tissue-specific differences in gene expression of MB, fission, and autophagy markers in the kidney and heart are in response to tissue-specific expression of other 5-HT_1 and 5-HT_2 receptors at 10 weeks. At 26 weeks of age, 5-HT_{1D}, 5-HT_{2A}, and 5-HT_{2C} receptor transcripts were upregulated in both the kidney and heart. This similar pattern in 5-HT receptor gene expression corresponds to elevated PGC-1α gene expression in both the kidney and heart at 26 weeks. Future studies will need to investigate the tissue-

dependent roles of 5-HT receptor signaling in MB in response to the absence of the 5-HT_{1F} receptor.

Having demonstrated that 5-HT_{1F} receptor-deficient mice are healthy, we investigated the effects of I/R- induced AKI on renal function and MB in 5-HT_{1F} receptor KO mice. The rationale for these experiments was supported by our previous findings that demonstrated stimulation of the 5-HT_{1F} receptor accelerated renal recovery following I/R-induced AKI and rescued mtDNA copy number in mice (5). An important role for the 5-HT_{1F} receptor in the onset and recovery of renal injury following AKI was demonstrated by sustained renal tubular injury and worsened tubular injury as measured by KIM-1 and NGAL protein expression in KO mice. These data are corroborated by the reduction in 5-HT_{1F} receptor gene expression following AKI in WT mice at 24 hr. However, there were no differences in renal cortical histology between injured WT and 5-HT_{1F} receptor KO mice. A possible explanation for similar proximal tubule necrosis and brush border loss is that both genotypes reached maximal histological injury at 24 hr.

The observation that renal injury was worsened in 5-HT_{1F} receptor KO mice was also associated with persistent suppression of MB. Our findings are supported by published results from our laboratory and other groups that have demonstrated that PGC-1α and MB are protective following I/R-induced AKI (25-27). We propose that loss of 5-HT_{1F} receptor dependent MB is the mechanism responsible for diminished PGC-1α and ETC protein expression observed in the 5-HT_{1F} receptor KO mice. Given that the generation of ATP primarily takes place at the ETC, it is unsurprising that the 5-HT_{1F} receptor KO mice exhibit renal cortical ATP depletion. It is also important to mention that 5-HT_{1F} receptor mice displayed elevated oxidative protein damage following I/R-induced AKI. Further studies are needed to fully understand the mechanisms by which the 5-HT_{1F} receptor is mediating oxidative stress however, we hypothesize that MB governed by the

5-HT_{1F} receptor is essential for the maintenance of proper mitochondrial homeostasis and the depletion of this receptor is sufficient to disrupt normal mitochondrial quality control mechanisms that mediate renal organ recovery.

This study provides insight for the role of 5-HT_{1F} receptor in modulating MB and mitochondrial homeostasis. Mitochondrial dysfunction is a critical pathophysiological mediator in various disease states, including, acute organ injury and cardiovascular disease (2, 28-31). The induction of MB to restore mitochondria number and function increases the energy supply needed for tissue repair. Thus, MB is a promising therapeutic strategy for many human pathologies. However, there are only a few, nontoxic compounds that selectively induce MB (2). This presents an opportunity to target 5-HT receptors for the treatment of human acute organ injuries and cardiovascular diseases. We have provided evidence that 5-HT_{1F}, and other 5-HT_1 and 5-HT_2 receptors are present in human renal and cardiac tissue. While classical functional roles of 5-HT_1 and 5-HT_2 receptors in the cardiovascular system include renal vascular dilation, vasoconstriction, and platelet aggregation (32). Future studies will be needed to understand the involvement of 5-HT receptors in MB and the pathogenesis of human acute organ injuries and cardiovascular disease.

In summary, these results provide the first evidence that the 5-HT_{1F} receptor regulates mitochondria in renal and cardiac tissue under physiological conditions. Our data also demonstrates that the 5-HT_{1F} receptor plays a protective role in I/R- induced AKI and this protection is mediated, through mitochondrial homeostasis and biogenesis.

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CHAPTER FOUR:

Summary, Contributions and Future Directions

SUMMARY OF CURRENT LITERATURE

Disruption of MB in AKI

Mitochondrial dysfunction, particularly within the renal tubular epithelium, has been implicated as a major contributor to the development and progression of AKI (1). Based on experimental and clinical studies of ischemic, septic and nephrotoxic AKI, disruption in mitochondrial homeostasis creates a pathogenic environment (increased ROS, apoptosis, calcium influx, ATP depletion), ultimately, preventing renal repair (2-4).

The main focus of our laboratory has been dedicated to understanding the role of MB following AKI as a potential mechanism and therapeutic target. MB is a multifactorial process, including transcription and translation of nuclear-and mitochondrial encoded proteins involved in oxidative phosphorylation and mitochondrial dynamics, to generate new, functional mitochondria (5). Our laboratory demonstrated suppression of MB in multiple models of AKI. In particular, Funk et al. observed reduced gene and protein expression of PGC-1α, as well as reduced activity as measured by increased acetylation in the renal cortex of mice subjected to I/R-induced AKI (6). As the master regulator of MB, reduced PGC-1α was associated with persistent decreased renal cortical expression of several respiratory proteins, nuclear-encoded NDUFB8 and ATP synthase β, and mitochondrial-encoded COXI and COXIV (6). These findings indicate persistent suppression of MB after renal ischemic injury and similar changes were observed in mouse models of glycerol-induced rhabdomyolysis, folic acid nephropathy and septic-induced AKI (6-8). These studies were further extended to demonstrate the link between

MB and mitochondrial dynamics and function. Disruptions in MB were also accompanied by up-regulation of mitochondrial fission regulator, Drp1 and caspase 3 cleavage, indicating mitochondrial fragmentation and apoptotic induction in I/R and glycerol-induced AKI (6). Further studies revealed the loss of in tubular FCCP-uncoupled oxygen consumption and ADP-stimulated respiration in isolated mitochondria of mice subjected to I/R-induced AKI, supporting the importance of MB in the setting of AKI (9).

It is important to note that changes in mitochondrial homeostasis have been documented in human AKI patients. Skeletal muscle biopsies from septic patients admitted to the intensive care unit exhibited decreased ATP content, reduced complex I activity, glutathione depletion and elevated markers of oxidative stress. In addition, mitochondrial swelling was observed in biopsy samples from patients that suffered I/R-induced AKI (10). In a recent study by Whitaker, et al. urinary mtDNA copy number was associated with increased risk of worsening of AKI progression in patients that developed AKI post-cardiac surgery (11). Taken together, these findings indicate that mitochondrial dysfunction and loss of mitochondrial number is a common entity in both the experimental and clinical setting. Thus, pharmacological targeting of MB represents a viable strategy to prevent both mitochondrial and renal dysfunction following AKI.

Our laboratory has explored the molecular mechanisms that are responsible for alterations in MB in cellular and animal models of renal oxidant and ischemic injury. These studies revealed that stimulation of the β_2-adrenergic receptor, a $G\alpha_s$-coupled GPCR, by formoterol restores respiratory protein expression, mitochondrial and renal function after I/R injury in mice (9). In addition, treatment with sildenafil, a cGMP-selective phosphodiesterase 5 inhibitor promotes increased MB and function following folic acid-induced AKI (7). These findings indicate that GPCRs and cGMP are potential mediators of MB signaling. One goal of the work discussed here was to further

characterize signaling events leading to the induction of PGC-1α and MB. We hypothesize that this work will increase the understanding of mechanisms underlying mitochondrial dysfunction and identify better therapeutic approaches for the treatment of renal injury.

Role of Serotonin in Mitochondrial Homeostasis and Biogenesis

Despite promising results in the identification of mitochondrial biogenic compounds, these studies only yield partial recovery of mitochondrial and renal function, so our laboratory further characterized pathways leading to MB. To this end, serotonin receptor activation was identified as an efficacious inducer of MB in cellular and animal models. Rasbach et al. identified the 5-HT_2 family of receptors as a regulator of MB (12). RPTC treated with the 5-HT_2 pan-agonist DOI increased FCCP-uncoupled respiration, respiratory protein expression, ATP generation and mitochondrial mass/number, indicating biogenesis of new, functional mitochondria in response to 5-HT_2 receptor activation (12). Additionally, DOI accelerated the recovery of mitochondrial function following oxidant injury in primary RPTC, further indicating the therapeutic potential of the 5-HT_2 receptor class (12). This study also revealed that the induction of MB in renal proximal cells is indeed dependent on PGC-1α, strengthening the need for the elucidation of pathways upstream of PGC-1α activation.

Additional work was performed to determine the specific 5-HT_2 receptor responsible for the observed biogenic effects of DOI. The 5-HT_2 receptor family is coupled to $G\alpha_s$ and comprised of three receptors, 5-$HT_{2A,B,C}$. Using a Seahorse Extracellular Flux assay, a phenotypic screen used for the identification of mitochondrial biogenic agents, our laboratory identified the 5-HT_{2C} receptor agonist CP-809,101 and 5-HT_{2C} receptor antagonist SC-242,084 as potent inducers of MB based on increased FCCP-uncoupled oxygen consumption (13). Both compounds increased PGC-1α mRNA and gene

expression of nuclear-and mitochondrial-encoded respiratory chain components in primary RPTC and similar changes were observed in mice (13).

It is intriguing that both an agonist and antagonist for the same receptor elicit the same mitochondrial biogenic response. To determine if CP-809,101 and SB-242,084 are acting through the 5-HT_{2C} receptor, both compounds were administered to mice lacking the 5-HT_{2C} receptor (13). In the absence of the 5-HT_{2C} receptor, NDUFB8 and ND1 were further elevated compared to wild-type control mice treated with each compound, indicating that the 5-HT_{2C} receptor is not responsible for the mitochondrial biogenic effect observed in the presence of CP-809,101 and SB-242,084. However, the 5-HT_{2C} receptor does seem to be involved in the maintenance of mitochondrial homeostasis in the renal cortex as the absence of the 5-HT_{2C} receptor reduced PGC-1α mRNA expression under physiological conditions (13). Further examination of this phenomenon, revealed that these compounds exert their effects through the 5-HT_{2A} receptor (13). These findings were confirmed utilizing RPTC that lacked expression of the 5-HT_{2A} receptor and no change in PGC-1α mRNA was noted with either drug (13). These results indicate that the 5-HT_{2A} receptor is responsible for the induction of MB in the renal cortex.

Although the pharmacological potential of 5-HT_{2A} receptor agonists and antagonists was disappointing, our laboratory continued to screen other 5-HT receptor agents for their ability to induce MB. This screening revealed that the LY334370 and LY344864, agonists of the $G\alpha_i$-coupled, 5-HT_{1F} receptor induced FCCP-uncoupled oxygen consumption rates in RPTC at nanomolar concentrations (14). Garrett. et al. also determined that both 5-HT_{1F} receptor agonists increased protein expression of NDUFB8, ATP Synthase β and COX1 and these changes were blocked in RPTC transfected with siRNA against the 5-HT_{1F} receptor, demonstrating that both agonists are indeed signaling through the 5-HT_{1F} receptor (14). It is important to note that knock down of the

5-HT_{1F} receptor resulted in decreased mitochondrial proteins, suggesting that the 5-HT_{1F} receptor is required for proper mitochondrial homeostasis under physiological levels (14). In mice, LY344864 increased renal cortical mRNA expression of PGC-1α, COX1 and NDUFB8, as well as mtDNA copy number. Of note, 5-HT_{1F} receptor was identified in a number of tissues, including cardiac and hepatic, and MB was also observed in these tissues following LY344864 treatment (14). The most intriguing aspect of this study is LY344864 accelerated the recovery of renal function following I/R-induced AKI. The LY344864 induced-restoration in renal function was associated with the recovery of renal mtDNA copy number (14). Interpretation of these data reveal the 5-HT_{1F} receptor should be considered a promising regulator of MB and a key target for treating renal injury.

When discussing the role of 5-HT receptors in modulating mitochondrial homeostasis, it is important to discuss the historical findings by Nebigil et al. detailing the significant role of the 5-HT_{2B} receptor in cardiac mitochondrial function and structural integrity (15). Specifically, their laboratory demonstrated that the ablation and overexpression of the 5-HT_{2B} receptor both lead to alterations in mitochondria. Overexpression of cardiac 5-HT_{2B} receptor lead to increased ventricular mitochondrial enzyme activities and increased mitochondrial proliferation lead to increased cardiac hypertrophy (16). In contrast, specific knockout of cardiac 5-HT_{2B} receptor lead to reduction in succinate dehydrogenase cytochrome oxidase activity and mitochondrial number as well as altered mitochondrial structure. In addition, these mice exhibited increased myocardial cell death (17). These studies further illustrate the role of 5-HT receptors in regulating cellular functions under both physiological and pathological conditions.

Pharmacological Induction of MB for the Treatment of Acute Organ Injury

Acute organ injuries in multiple organ systems share common pathophysiological features, including vascular disturbances, inflammation and mitochondrial dysfunction. Experimental strategies have targeted each of these pathological events with hopes to gain a better understanding of the major contributors of acute organ dysfunction and to identify better treatment strategies to restore organ function following insult. Of these studies, modulation of mitochondrial function and number has proven to be one of the most promising approaches. This section will discuss the variety of potential drug targets of MB and the consequent improvement in mitochondrial homeostasis in multiple tissues to demonstrate the true effectiveness of inducing MB in acute organ injury.

AMPK: AMPK is a ubiquitously expressed heterotrimeric kinase that acts as a highly conserved energy sensor and participates in the regulation of energy-generating and consumption pathways. AMPK triggers increases in glucose uptake, oxidative phosphorylation, autophagy, and MB. AMPK has been reported to directly phosphorylate PGC-1α at two sites, activating its transcription (18). An alternative mechanism by which AMPK may activate PGC-1α function is via deacetylation catalyzed by the nicotinamide adenine dinucleotide (NAD)-dependent deacetylase sirtuin (SIRT) 1(19). Stimulation of AMPK following ATP depletion in rats enhanced NRF-1 binding activity and subsequently restored mitochondrial density. Pharmacological activation of AMPK has been observed with multiple compounds, including 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) (20, 21). AICAR is an adenosine analog AMPK activator, which has shown positive effects following cardiac and renal I/R injury through enhanced glucose uptake and SIRT1 expression, respectively (22, 23). AICAR has also been successful in chronic disease models, such as type II diabetes, by preventing insulin

resistance in multiple tissues, as well as in AD through decreases in amyloid-β protein levels (24-26).

ERK1/2: MAPKs are serine-threonine kinases that mediate intracellular signaling associated with a variety of cellular activities, including cell proliferation, differentiation, survival, death, and transformation. As discussed above, ERK1/2, a member of the MAPK family, has been implicated as a negative regulator of MB (27) as ERK1/2 reduces PGC-1α and its downstream targets in a number of disease models. For example, ERK1/2 activation impaired mitochondrial function, as evidenced by altered mitochondrial dynamic processes and reduced mitochondrial membrane potential in an animal model of Alzheimer's disease (28). Additionally, ERK1/2 activation preceded the initial decrease in renal PGC-1α mRNA and loss of renal function in a mouse model of I/R-induced AKI (27).

ERK1/2 is thought to be the only substrate for mitogen-activated protein kinase kinase (MEK1/2) phosphorylation; therefore, pharmacological inhibition of ERK1/2 can be accomplished using MEK1/2 inhibitors. Treatment with the MEK1/2 inhibitor U0126 was found to rescue hippocampal PGC-1α, TFAM, and NRF-1 protein levels following amyloid-β injections in rats (29). Additionally, our laboratory has reported that inhibition of ERK1/2 using trametinib not only attenuates the early decrease in PGC-1α, but also prevents decreased renal function following I/R-AKI (27). These studies provide evidence that targeting negative regulators of MB is an effective strategy to induce MB and restore organ function following injury.

cAMP/PKA/CREB Pathway: PDE inhibitors tightly regulate levels of cAMP, thus, these compounds are often used to activate the cAMP/PKA/CREB pathway. Rolipram and cilostazol are cAMP-selective PDE inhibitors that have been found to induce PGC-1α

and mitochondrial activity in multiple cellular and animal models. For example, rolipram increased PGC-1α deacetylation, PGC-1α mRNA expression and mtDNA content in skeletal muscle isolated from mice (30). Similar changes were observed in cultured endothelial cells treated with cilostazol (31). In addition, rolipram and cilostazol demonstrated protection in preclinical models of neurodegenerative disease (32, 33).

Sirtuins: The SIRTs are a family of proteins that act predominately as NAD-dependent deacetylases. As mentioned above, PGC-1α is activated through deacetylation, which primarily occurs via its interaction with SIRT1. SIRT1 also controls the acetylation of forkhead box O (FOXO) transcription factors, acetylation status of FOXO directs it to certain targets, such as the PGC-1α promoter (34, 35). Studies revealing SIRT1-induced activation of PGC-1α led to the interest in nutraceutical and pharmacological activators of SIRT1.

Resveratrol and other natural products, such as the isoflavones found in soybeans, the flavonoids such as quercetin and green tea polyphenols found in fruits and cocoa, respectively, have been shown to increase SIRT1-mediated MB and metabolic functions (36-39). Interestingly, quercetin has been shown to increase PGC-1α expression and mtDNA copy number in various brain regions of rats exposed to aluminum, a widely distributed element linked to several neurological diseases including Alzheimer's and Parkinson's disease.(40, 41). PGC-1α activation using green tea polyphenols rescued reductions in respiratory chain proteins and mtDNA content in a rat model of cyclosporine A-induced nephrotoxicity, and consequently, attenuating kidney injury and improving renal function. These data implicate natural products as a promising therapeutic strategy for toxicant-induced mitochondrial suppression to promote tissue repair and regeneration (42). Identification of natural SIRT1 activators encouraged the search for pharmacological inducers of SIRT1. As a result, a number of SIRT1 activators

were developed, including SRT1720, SRT1460, and SRT2104. Specifically, SRT1720 demonstrated protective effects across a variety of disease states in mice, including renal ischemic injury and MI, through activation of SIRT1/PGC-1α (43, 44).

In summary, preclinical and human studies indicate that the induction of MB via various pathways promote functional recovery in a number of disease states, including metabolic disorders, I/R injuries, neurodegenerative and cardiovascular pathologies. Unfortunately, relatively few drugs have been identified for the induction of MB, and such drugs are often functionally promiscuous. Therefore, a significant amount of work is still needed to identify safe and efficacious compounds. The above list, including mitochondrial biogenic agents identified by our laboratory provide researchers and clinicians with valuable tools for developing mitochondrial biogenic based strategies for therapeutic intervention for both acute and chronic diseases.

CONTRIBUTIONS TO THE FIELD

Mechanisms Underlying the Induction of MB

Although our laboratory and others have identified a number of mitochondrial biogenic targets, there has been very few studies aimed at determining the molecular mechanism mediating this effect. Specifically, we explored the signal transduction mechanism linking the 5-HT_{1F} receptor to MB. As mentioned earlier, the 5-HT_{1F} receptor is a $G\alpha_i$-coupled receptor. As the Gβγ heterodimer mediates much of $G\alpha_{i/o}$ signaling (45), its role was an important aspect to consider when investigating the 5-HT_{1F} receptor-mediated MB pathway. Additionally, there was some evidence indicating that the eNOS/cGMP/PKG pathway is involved in regulating PGC-1α and MB (7, 46, 47).

To explore this possibility, we utilized the Seahorse Extracellular Flux Assay to determine the key regulatory components mediating LY344864-induced cellular respiration as a marker for MB. As stated earlier LY344864 is a potent and specific agonist of the 5-HT_{1F} receptor. LY344864 increased FCCP-uncoupled oxygen consumption in RPTC, which was prevented when RPTC were pretreated with inhibitors of Gβγ heterodimer, Akt, NOS, sGC and PKG. To confirm that the changes observed in FCCP-uncoupled oxygen consumption were indicative of changes in MB, mtDNA copy number was assessed and similar findings were observed. These findings indicate that Gβγ heterodimer, Akt, NOS, sGC (cGMP) and PKG are key components of the 5-HT_{1F} receptor induced MB pathway.

Since we identified potential mediators of 5-HT_{1F} receptor-induced MB, we then elucidated the sequence of events within this pathway that result in MB following 5-HT_{1F} receptor stimulation. The sequence of events identified were agonist stimulation of 5-HT_{1F} receptor leading to Gβγ dependent activation of Akt/eNOS/PKG/PGC-1α.

To investigate the potential for additional signaling events downstream of 5-HT_{1F} receptor that stimulate MB, we focused on the MAPK pathway since ERK1/2 has been implicated as a negative regulator of PGC-1α and MB (27). Interestingly, LY344864 decreased ERK1/2 phosphorylation in RPTC. Next, we examined the upstream signaling events that lead to diminished ERK1/2 phosphorylation, especially since the canonical MAPK pathway is independent of the PI3K/Akt/eNOS signaling axis. We determined Gβγ heterodimer and Akt activation are responsible for the suppression of ERK1/2 phosphorylation, through the direct inhibition of c-raf-MEK1/2 and ERK1/2 phosphorylation.

Overall, this study demonstrated that 5-HT_{1F} receptor-mediated MB occurs through dual mechanisms dependent upon Gβγ heterodimer activation. Specifically, Gβγ heterodimer dependent signaling is activating a known mitochondrial biogenic stimulatory pathway, Akt/eNOS/cGMP/PKG/PGC-1α, while simultaneously suppressing an inhibitory mitochondrial biogenic pathway, c-raf/MEK/ERK/FOXO3a. We hypothesize that the dual mechanisms are responsible for the efficacy of LY344864 induction of MB, thus, providing an explanation for the robust mitochondrial biogenic effects exerted by LY344864 under physiological and pathological conditions. To our knowledge, this is the first study to elucidate a 5-HT_{1F} receptor-mediated MB pathway. This novel mechanism of MB provides a better understanding of the cellular events leading to increased mitochondrial number and identifies additional therapeutic targets for diseases characterized by mitochondrial dysfunction.

5-HT_{1F} receptor as an in vivo regulator of MB

Given the beneficial effects of 5-HT_{1F} receptor stimulation described by Garrett et al., we were interested in understanding the role of the 5-HT_{1F} receptor in the regulation of mitochondrial and renal biogenesis and function under physiological and pathological

conditions. To explore this relationship, we utilized 5-HT_{1F} receptor KO mice. Since a significant increase in renal cortical MB markers was observed in naïve mice following LY344864 administration, we postulated that the absence of the 5-HT_{1F} receptor would lead to the suppression of PGC-1α and its downstream targets. However, a significant increase in renal cortical genes that encode for nuclear-and mitochondrial-encoded respiratory chain proteins, as well as mtDNA copy number was observed. In addition, we detected increased mRNA expression in Drp1 and Pink1 in the renal cortex of 5-HT_{1F} receptor KO mice. It is important to note that these observations were made in young (10-week old) WT and 5-HT_{1F} receptor KO mice. Based on the established role of mitochondria in age-related pathological processes, we assessed the same parameters for mitochondrial homeostasis in aged (26-week old) WT and 5-HT_{1F} receptor KO mice, and, surprisingly, PGC-1α mRNA expression and mtDNA copy number remained elevated in the renal cortex as 5-HT_{1F} receptor KO mice aged. In summary, 5-HT_{1F} receptor KO mice exhibited increased expression of genes involved in the proper maintenance of mitochondrial homeostasis, suggesting that renal cortical signaling mechanism(s) have the ability to initiate an adaptive response to limit deleterious effects in the absence of the 5-HT_{1F} receptor under physiological conditions.

We also assessed the gene expression of mitochondrial homeostasis markers in left ventricle tissue since the 5-HT_{1F} receptor has been shown to stimulate cardiac MB and it is an important contributor to and target of renal dysfunction. In contrast to changes observed in the renal cortex of 5-HT_{1F} receptor mice, we observed decreased mRNA expression of respiratory chain components, fission and autophagy markers in the left ventricles of young 5-HT_{1F} receptor KO mice. In addition, the majority of these markers returned to WT levels as the 5-HT_{1F} receptor mice aged. It is noteworthy to mention that cardiac PGC-1α mRNA was elevated in the aged 5-HT_{1F} receptor mouse, indicating that

PGC-1α is involved in the rescue of mitochondrial homeostasis markers in aged 5-HT_{1F} receptor KO mice. Taken together, these findings revealed distinct patterns of mitochondrial gene expression in a tissue-specific manner in the absence of the 5-HT_{1F} receptor. Specifically, these results strongly support the idea that activation of compensatory mechanisms occurs in the renal cortex but that, this response seems to be absent or insufficient in the left ventricle of 5-HT_{1F} receptor KO mice.

After determining 5-HT_{1F} receptor-mediated tissue-specific differences in mitochondrial homeostasis markers, we focused on elucidating the mechanisms responsible for this observation. To this end, we assessed mRNA expression of other $5HT_1$ and 5-HT_2 receptors in naïve WT and 5-HT_{1F} receptor KO mice. Interesting, we observed distinct renal cortical and left ventricle mRNA expression patterns of 5-HT_{2A} and 5-HT_{2B} receptors, which have both been implicated as regulators of MB and/or function. Specifically, 5-HT_{2A} receptor gene expression is reduced and 5-HT_{2B} receptor gene expression is elevated in the renal cortex of young 5-HT_{1F} receptor KO mice. In contrast to these findings, increased 5-HT_{2A} receptor gene expression and decreased 5-HT_{2B} receptor gene expression was observed in aged 5-HT_{1F} receptor KO mice. We hypothesize that the differences in 5-HT_{2A} and 5-HT_{2B} receptor expression represents alterations in the signaling pathways downstream of these receptors and are involved, at least in part, in the differential changes in renal cortical and left ventricle mitochondrial homeostasis markers in 5-HT_{1F} receptor KO mice. It is noteworthy to mention that the expression of 5-HT_1 and 5-HT_2 receptors has been detected in human renal and cardiac tissue, highlighting the therapeutic potential for pharmacological intervention following renal and cardiac organ injuries. Further studies are warranted to confirm the physiological significance of tissue-specific 5-HT receptor signaling in the absence of the 5-HT_{1F} receptor.

We next focused on the role of the 5-HT_{1F} receptor in renal and mitochondrial homeostasis following I/R-induced AKI. While 5-HT_{1F} receptor KO mice are completely healthy under physiological conditions, we hypothesized that depletion of the 5-HT_{1F} receptor would lead to worsened outcomes under stress. While renal function, as measured by serum creatinine and BUN, of the 5-HT_{1F} receptor KO mice was no different of that of the WT mice 24 hr following I/R injury, the more sensitive markers renal tubular injury (KIM-1, NGAL) revealed that renal damage was greater in the 5-HT_{1F} receptor mice, suggesting that the 5-HT_{1F} receptor is involved in the initiation of tubular injury and, in turn, is likely to elicit renal protective effects under stress.

Earlier studies from our laboratory demonstrated that 5-HT_{1F} receptor stimulation accelerated the recovery of renal function and MB in the setting of I/R-induced AKI. Thus, we sought to determine if the absence of the 5-HT_{1F} receptor would prevent renal recovery. As expected, 5-HT_{1F} receptor KO mice failed to recover renal function by 144 hr following the renal I/R insult, compared to the partial recovery of renal function observed in WT mice. In fact, renal recovery in WT mice was approximately 50% greater compared to 5-HT_{1F} receptor KO mice. In addition, failed renal recovery was associated with elevated tubular injury in 5-HT_{1F} receptor KO mice post-I/R injury. Taken together, these findings support the notion that the 5-HT_{1F} receptor has a meaningful role in facilitating repair and/or regenerative mechanisms necessary for renal function recovery.

As discussed earlier, renal recovery following AKI depends on adequate mitochondrial homeostasis and function. Therefore, impaired renal recovery in the absence of the 5-HT_{1F} receptor may thus be linked to disruption in MB. Renal cortical PGC-1α and its downstream targets, nuclear-and-mitochondrial proteins of the respiratory chain were persistently suppressed in 5-HT_{1F} receptor KO mice compared to WT mice subjected to renal I/R injury. These findings were also associated with persistent depletion of renal

cortical ATP production in 5-HT_{1F} receptor KO mice. Interpretation of these findings suggest that disrupted MB, and subsequent impairment of mitochondrial function are together a potential explanation for worsened tubular injury and renal function in the 5-HT_{1F} receptor mice. These findings are consistent with previous reports from our laboratory demonstrating that disruption of MB is closely associated with worsened tubular injury and renal function following I/R-induced AKI.

Because PGC-1α is also involved in the regulation of additional mitochondrial processes, such as ROS-detoxifying mechanisms, we measured SOD2 protein expression as it participates in reducing ROS in the mitochondria. We detected a significant reduction in SOD2 protein in 5-HT_{1F} receptor KO mice and this was associated with increased oxidative protein damage in the renal cortex of 5-HT_{1F} receptor mice following I/R-induced AKI. These observed changes were absent in WT mice. Further studies are needed to identify the mechanism by which the 5-HT_{1F} receptor modulates oxidative stress following renal I/R injury. However, we hypothesize that reduced PGC-1α expression and signaling in the absence of the 5-HT_{1F} receptor ultimately impairs the ability for MB pathways to induce expression of proteins required for both oxidative phosphorylation and ROS scavenging programs. Overall, these findings reveal that the 5-HT_{1F} receptor exerts renal protective effects by modulating MB and function in the setting of I/R-induced AKI.

FUTURE DIRECTIONS

5-HT_{1F} Receptor Mediated MB Signaling

While the pathway of 5-HT_{1F} receptor mediated MB was elucidated in the scope of this project, there are additional questions that remain unanswered. We were surprised to observe tissue-specific differences in the expression of mitochondrial homeostasis markers in the absence of the 5-HT_{1F} receptor. We hypothesized that signaling of other 5-HT receptors is involved in mediating this differential effect. However, we did not further pursue the physiological relevance of 5-HT receptor signaling in mitochondrial homeostasis. Additional experiments should investigate the role of 5-HT receptors in mediating mitochondrial homeostasis in the absence of the 5-HT_{1F} receptor as this may lead to identification of novel therapeutic targets. One of the most intriguing future experiments that should be performed is exploring the downstream signaling events contributing to the adaptive response in the kidney and to determine why this is not present or sufficient in the heart. To this end, we measured renal cortical ERK1/2 and Akt phosphorylation in WT and 5-HT_{1F} receptor KO mice. ERK1/2 and Akt phosphorylation was elevated in the absence of the 5-HT_{1F} receptor (Fig. 4.2). It would be interesting to conduct additional studies to investigate if the change in Akt and ERK1/2 phosphorylation status is contributing to the observed increase in mitochondrial homeostasis markers and to compare levels Akt and ERK1/2 phosphorylation in the left ventricle. We predict that the pattern of expression will differ from that of the renal cortex, potentially explaining the difference between renal cortical and left ventricular mitochondrial homeostasis in the absence of the 5-HT_{1F} receptor. Together, these results could provide additional information on the mechanisms regulating MB.

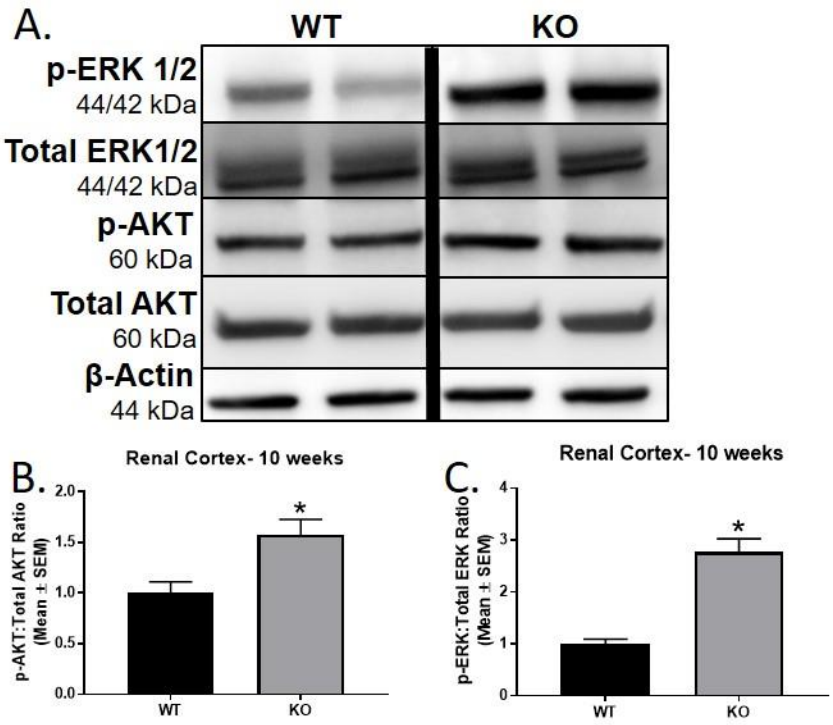

Figure 4.1: Renal cortical Akt and ERK phosphorylation is increased in the absence of the 5-HT$_{1F}$ receptor at 10 weeks. Total protein was harvested from the renal cortex of WT and 5-HT$_{1F}$ receptor KO mice. Immunoblot analysis of renal cortical Akt and ERK phosphorylation (A) was assessed and normalized to total Akt and ERK, respectively. Densitometry analysis of Akt (B) and ERK(C) phosphorylation. Data are reported as mean ± SEM, n = 4. * indicate statistically significant differences compared to WT controls ($p<0.05$).

The Role of the 5-HT_{1F} Receptor in Mitochondrial Homeostasis

The above findings present a number of exciting questions that deserve attention to fully understand the role of the 5-HT_{1F} receptor not only in MB, but also in mitochondrial homeostasis. In an I/R-induced model of AKI, we identified 5-HT_{1F} receptor-dependent activation of MB as an essential mechanism for renal recovery. We have been particularly interested in whether this pathway might involve the regulation of mitochondrial dynamics and mitophagy, that is, selective mitochondrial autophagy. While the generation of new, functional mitochondria is imperative to drive energy-dependent repair and renal processes in the face of injury, perhaps prompting the clearance of damaged and dysfunctional mitochondria is also important to prevent deleterious events, such as excessive ROS generation. Additionally, numerous reports have demonstrated that the balance of MB and mitophagy is required for proper energy metabolism under both physiological and pathological conditions.

To determine, if mitophagy is being modulated in response to LY344864-mediated MB in RPTC, we assessed a number of proteins that comprise the autophagy/mitophagy signal transduction pathway. At 24 hr, an increase in Akt/mTOR/ULK1 (S757) signaling pathway was observed by measuring the phosphorylation status of these proteins (Fig. 4.2 A-D). This pathway has been shown to inhibit autophagy/mitophagy. Next, we assessed components of the autophagic (LC3BII and p62) and mitophagic (Pink1) machinery following LY344864 treatment, however, no change in protein expression was observed in any of these markers at 24 hr (Fig. 4.2E-F). It is noteworthy to mention that at this same time point, 24 hr, LY344864 increases MB (cellular respiration and respiratory complex proteins). Given these results in naïve RPTC, we postulate that mitophagy signaling is not needed in uninjured RPTC and that suppression of mitophagy may further promote increased mitochondrial content. Further work using both genetic

and pharmacological approaches are warranted to determine the signaling mechanism contributing to 5-HT_{1F} receptor-mediated mitophagy signaling.

While we have elucidated the signaling mechanism responsible for 5-HT_{1F} receptor-mediated MB in RPTC, the mechanism mediating the degradation of mitochondrial proteins following LY344864-induced MB stimulation is still unknown. To address this, a series of wash-out experiments were performed in the presence and absence of the lysosomal inhibitor, chloroquine. Following a single dose of LY344864, nuclear-and mitochondrial-encoded proteins are increased at 24 hr and returned to control levels by 48 hr in RPTC. We demonstrate lysosomal inhibition prevents the degradation of mitochondrial proteins following LY344864-induced MB as evidenced by the persistent and potentiated increase in NDUFB8 and COX1 protein expression at 48 hr (Fig. 4.3A, B). According to these preliminary results, lysosomal degradation may responsible for mitochondrial protein degradation following MB. Further work is necessary to fully understand the mechanisms involved in mitochondrial protein degradation following MB.

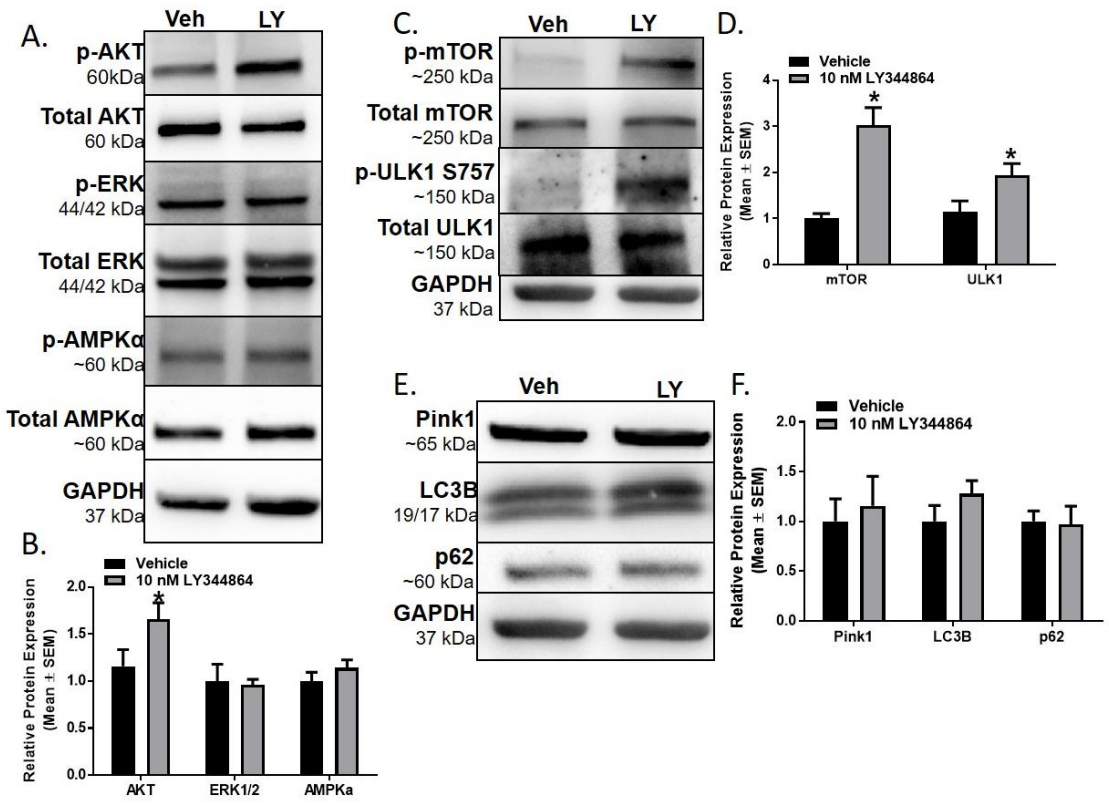

Figure 4.2: LY344864 suppresses autophagy/mitophagy signaling in naïve RPTC. Total protein was harvested from RPTC. To assess autophagy/mitophagy signaling, immunoblot analysis of Akt (A), ERK (A), AMPKα (A), mTOR (C) and ULK1 (Ser757) (C) phosphorylation were measured and normalized to total Akt, ERK, AMPKα, mTOR, ULK1, respectively. Densitometry analysis of Akt, ERK and AMPKα (B). Densitometry analysis of mTOR and ULK1 (D). Proteins involved in autophagy/mitophagy, Pink1, LC3BII and p62 were also assessed using immunoblot analysis. Densitometry of Pink1, LC3BII and p62 (F). Date reported as mean ± SEM, n = 4.* indicate statistically significant differences compared to vehicle treated controls($p<0.05$).

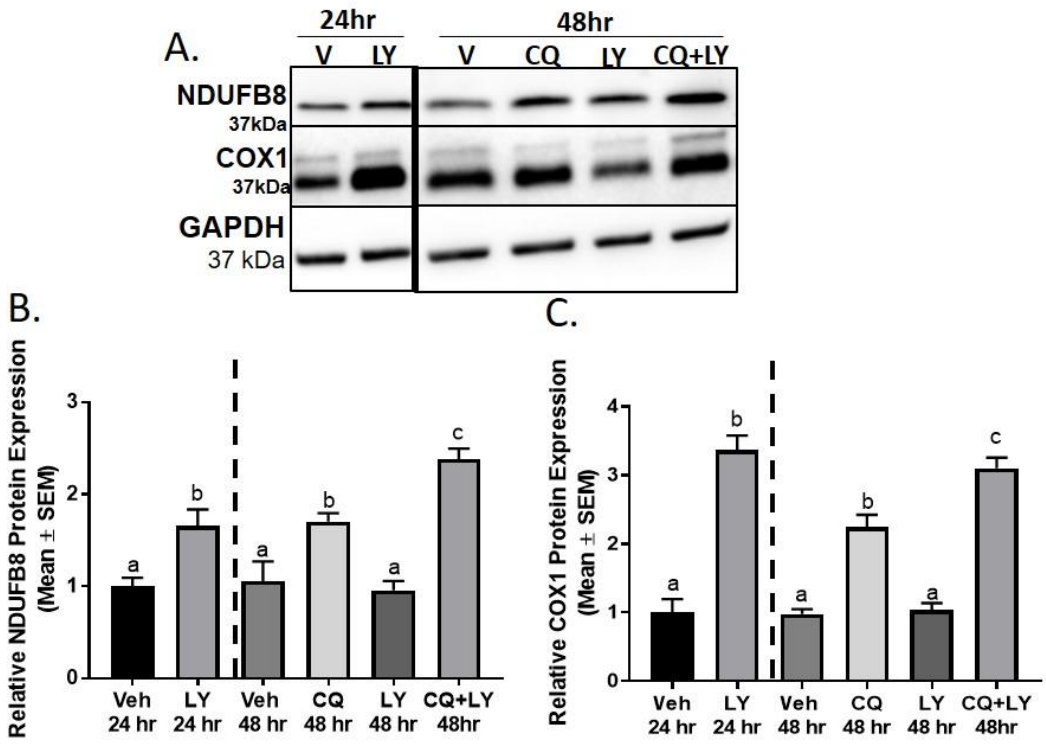

Figure 4.3: 5-HT$_{1F}$ receptor stimulation is required for sustained MB and degradation following MB is lysosomal-dependent. RPTC were pretreated with chloroquine or vehicle control, a lysosomal inhibitor, and then exposed to LY344864 or vehicle control for 24 or 48 hr. Total protein was harvested from RPTC and was subjected to immunoblot analysis. NDUFB8 and COX1 protein expression was assessed (A). Densitometry analysis of NDUFB8 and COX1 (B, C). Data reported as mean ± SEM, n = 4. Different superscripts indicate statistically significant differences ($p<0.05$).

Studies conducted in both cellular and animal models of renal injury have documented that the induction of autophagy is renal protective (48, 49). In fact, inhibition of autophagy has proven to worsen renal and mitochondrial outcomes following I/R-induced AKI (50). Our laboratory reported significant mitophagy, selective mitochondrial autophagy, as evidenced by colocalization of mitochondria and lysosomes following exposure to the oxidant TBHP in RPTC (51). These studies also revealed that PGC-1α protein expression was up-regulated for up to 3 days following TBHP exposure, and this was accompanied by elevated basal and uncoupled oxygen consumption rates (51). Rasbach et al. concluded that elevation of PGC-1α was responsible for the restoration of mitochondrial function by the generation of new mitochondria to compensate for the mitochondrial damage induced by TBHP.

To test the hypothesis that LY344864 is also a modulator of mitochondrial dynamics, I performed this same experimental setup with TBHP treated RPTC in the presence and absence of LY344864. It should be noted that I did not detect any changes in mitochondrial dynamic markers in naïve RPTC treated with LY344864 (data not shown). As expected PGC-1α protein was elevated following TBHP treatment of RPTC, however, mitochondrial fusion protein, Mfn2 and phosphorylation of Drp1 at Serine 637 was reduced (Fig. 4.4A-D), indicating enhanced mitochondrial fission. RPTC treated with LY344864 following TBHP treatment exhibited further increase in PGC-1α protein expression and enhanced Mfn2 expression and Drp1 Serine 637 phosphorylation. In addition, mtDNA copy number was also markedly increased in LY344864 treated RPTC following TBHP exposure compared to RPTC treated with TBHP alone. Taken together these findings provide strong evidence that 5-HT_{1F} receptor stimulation not only increases mitochondrial content but may also drive the formation of mitochondrial networks, both of which are considered beneficial for cellular survival (52). Based on

these findings and discovery of LY344864 suppressing autophagy/mitophagy signaling in naïve RPTC, future experiments are warranted to determine if and how LY344864 is modulating mitochondrial dynamics and mitophagy following injury.

To set the foundation for this aim, a preliminary experiment was performed with a similar experimental design as above and observed that, in LY344864 treated RPTC compared to RPTC treated with TBHP alone, there was a marked increased in phosphorylation of AMPKα and ULK1 (Serine 555) (Fig. 4.5A-C), which are key signaling events in autophagy signaling. In addition, greater expression of LC3BII and p62 was observed in RPTC treated with LY344964 following TBHP exposure (Fig. 4.6 A-C). These data reveal that LY344864 is indeed increasing autophagic flux, in turn, mitophagy may also be enhanced. Additionally, an autophagic flux assay utilizing chloriquine, a lysosomal inhibitor, was performed to confirm that LY344864-induced increase in autophagic markers following TBHP exposure were indeed due to increased autophagy (Fig. 4.6 D, E). Further examination of mitophagy markers such as Pink1 will resolve if 5-HT_{1F} receptor has a role in regulating mitophagy under stress conditions. It should be noted that immunoblot analysis of autophagy and mitophagy markers may not be the best approach in assessing these processes. Alternative approaches such as imaging should be employed in future experiments to accurately elucidate LY344864-mediated effects on autophagy/mitophagy under pathological conditions. It would be interesting to utilize the 5-HT_{1F} receptor KO mice subjected to renal I/R injury to further elucidate the role of 5-HT_{1F} receptor in mitochondrial homeostasis.

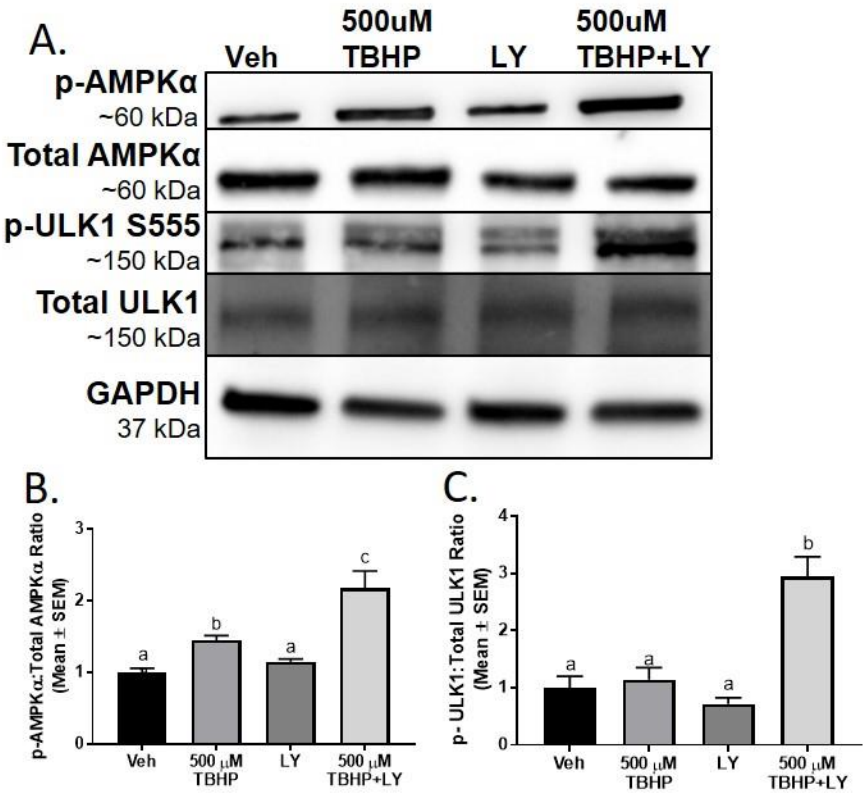

Figure 4.4: LY344864 increases PGC-1α protein expression and rescues markers of mitochondrial dynamics following oxidant injury in RPTC. RPTC were pretreated with TBHP or vehicle control for 45 min, then exposed to LY344864 or vehicle control for 24 hr. Total protein was isolated from RPTC and subjected to immunoblot analysis. PGC-1a, Mfn2 and DRP1 phosphorylation (A) were assessed. Densitometry analysis of PGC-1a, Mfn2 and Drp1 phosphorylation (normalized to total Drp1) (B-D). Data reported as mean ± SEM, n = 3. Different superscripts indicate statistically significant differences ($p<0.05$).

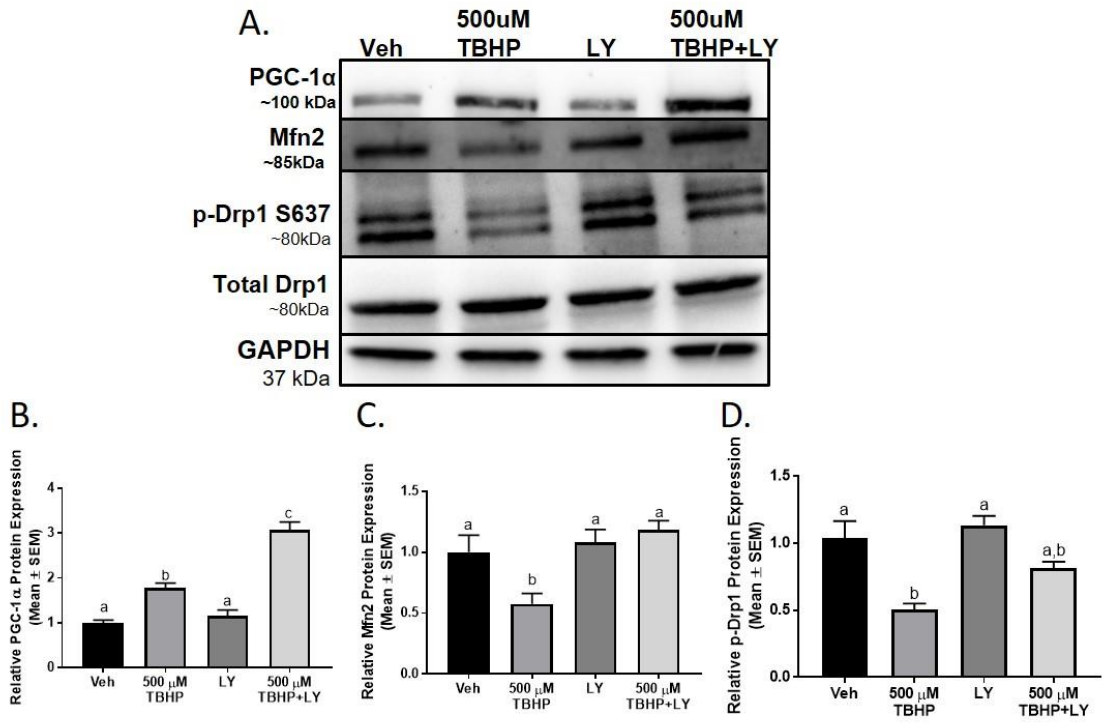

Figure 4.5: 5-HT$_{1F}$ receptor stimulation activates a stimulatory autophagy/mitophagy pathway following oxidant injury in RPTC. RPTC were pretreated with TBHP or vehicle control for 45 min, then exposed to LY344864 or vehicle control for 24 hr. Total protein was isolated from RPTC and subjected to immunoblot analysis. AMPKα and ULK1 (Ser555) phosphorylation (A) were assessed. Densitometry analysis of AMPKα and ULK1 phosphorylation, normalized to total AMPKα and ULK1, respectively (B, C). Data reported as mean ± SEM, n = 3. Different superscripts indicate statistically significant differences ($p<0.05$).

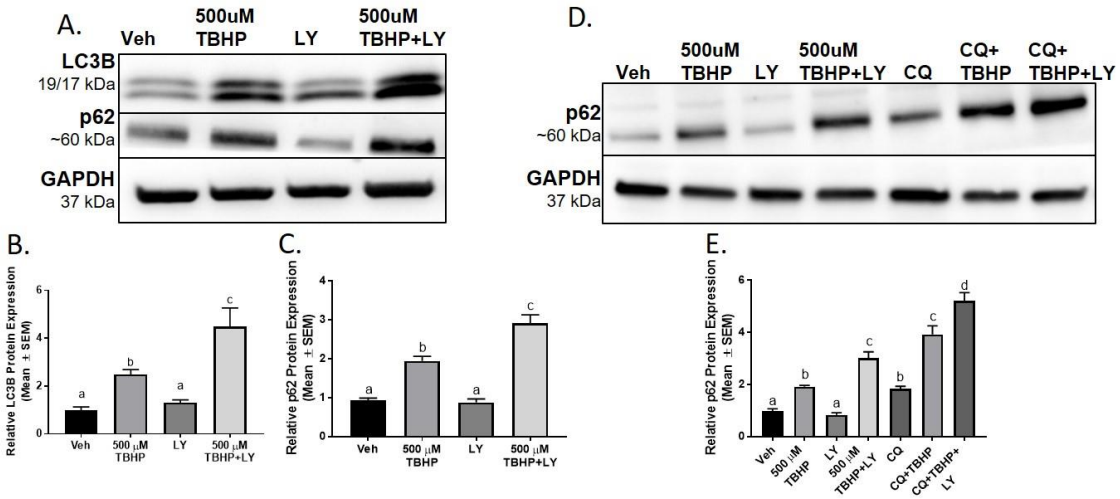

Figure 4.6: 5-HT$_{1F}$ receptor stimulation increases autophagic flux following oxidant injury in RPTC.RPTC were pretreated with TBHP or vehicle control for 45 min, then exposed to LY344864 or vehicle control for 24 hr. Total protein was isolated from RPTC and subjected to immunoblot analysis. LC3BII and p62 (A) were assessed. Densitometry analysis of LC3BII and p62 (B,C) TBHP and LY344864 treated RPTC were pretreated with chloroquine to assess autophagic flux. Protein isolated from these RPTC were subjected to immunoblot analysis and p62 was assessed (D). Densitometry analysis of p62 (E). Data reported as mean ± SEM, n = 3-4. Different superscripts indicate statistically significant differences ($p<0.05$).

The Role of the 5-HT_{1F} Receptor in Endothelial Homeostasis

Recent studies have documented the role of the renal vasculature in the progression from AKI to chronic kidney disease. The endothelial cells have been identified as a target of injury, as AKI results in a marked reduction in microvasculature. Renal injury may directly damage the renal vasculature and alter its activity; such as alter vascular responsiveness, barrier function, coagulation cascades, and inflammatory processes (53-55). Unfortunately, there are many unanswered questions about the mechanisms of renal endothelial repair and regeneration. Particularly, there are very few studies on the role of angiogenesis, or new vessel formation, which is required for oxygen and nutrient delivery during tissue repair, after AKI, and how modulation of angiogenesis may lead to accelerated renal repair.

Based on our previous findings that MB is renal protective as it accelerates the recovery of renal function and tubular injury, our laboratory has recently begun to document the role of MB in modulating angiogenic responses in endothelial cells. Our preliminary findings revealed that MB and homeostasis are required for angiogenic responses. Thus, additional experiments should test the hypothesis that MB in the renal endothelium will increase kidney function after injury. Our preliminary data demonstrates that targeting the 5-HT_{1F} receptor is a promising strategy which is unsurprising as the 5-HT receptors are well-documented in modulating vascular functions. Specifically, our findings indicate the expression of the 5-HT_{1F} receptor in the endothelial cell and in the absence of the 5-HT_{1F} receptor the renal vasculature is significantly reduced. Additionally, stimulation of the 5-HT_{1F} receptor increases MB in endothelial cells and this was associated with enhances angiogenesis *in vitro* and *in vivo*. Endothelial 5-HT_{1F} receptor stimulation under hypoxic conditions also increased cellular proliferation and repair. Overall our preliminary findings suggest that 5-HT_{1F} receptor stimulation of MB

and the subsequent activation of angiogenesis is protective in endothelial cells under stress conditions. Future studies using our recently developed 5-HT_{1F} receptor KO mice are required to fully understand the role of the 5-HT_{1F} receptor and its downstream signaling events in regulating endothelial cell function physiological and pathological conditions. Additionally, future studies are needed to explore the role of 5-HT_{1F} receptor agonists in promoting renal vascular recovery and function after I/R injury in mice. It is noteworthy to mention that our laboratory is in the process of developing proximal tubule and endothelial specific 5-HT_{1F} receptor KO mice, which will aid in the experimental design of all future studies discussed in this section.

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APPENDIX

Disrupted Mitochondrial Homeostasis and Inflammation in Peri-infarct Cortex and Striatum Following Stroke: A Window for Therapeutic Intervention

ABSTRACT

The goal of these studies is to establish the relationship between acute mitochondrial dysfunction, inflammation and behavioral outcomes following a unilateral ischemic stroke. Rats received unilateral stroke and were tested on sensitive behavioral tasks. Animals were then sacrificed at 24h, 48h and 144 h to determine expression of key mitochondria factors and indicators of inflammation, apoptosis and regenerative processes in ipsilesion cortex and striatum. Rats with stroke induced-behavioral deficits had sustained, 144 h post-lesion, decreases in mitochondrial-encoded electron transport chain proteins NADH dehydrogenase subunit-1 and cytochrome *c* oxidase subunit-1 (mRNA and protein) and mitochondrial DNA content in the remaining perilesion cortex. Uncoupling-protein-2 gene expression, but not superoxide dismutase-2, remained elevated in cortex and striatum at this time. Cortical inflammatory cytokine, interleukin-6, was increased early and followed by increased macrophage marker F4/80 after stroke. Cleaved caspase-3 activation, a marker of apoptosis, was elevated in cortex and growth associated protein -43 was elevated in the cortex and striatum six days post-lesion. We found a relationship between three disrupted pathways, identified as (1) sustained loss of mitochondrial proteins and mitochondrial DNA copy number in the cortex linked to decreased mitochondrial gene transcription; (2) early inflammatory response mediated by interleukin- 6 followed by macrophages; (3) apoptosis in conjunction with the activation of regenerative pathways. The stroke-induced spatial and temporal profiles lay the foundation to target neuroprotecitve and behavioral therapeutics.

INTRODUCTION

Stroke is currently the fourth leading cause of death and the leading cause of long-term disability in the United States (1). Annually, stroke is responsible for 130,000 deaths with an estimated cost of 34 billion dollars for medications, health care, and disability services (2, 3). Ischemic strokes make up 87% of all strokes [2] and treatment for ischemic stroke patients is limited to TPA, which has a small window of effectiveness (<4hr post-stroke) and several severe side effects. Therefore, there is a need to improve treatment options to decrease the morbidity and mortality associated with cerebral ischemia.

Within minutes to hours after injury, apoptosis starts to occur, normally due to calcium influx and mitochondria dysfunction (4). Degeneration of distal axons, also known as Wallerian degeneration occurs days to weeks following injury due to onset of deleterious metabolic pathways which leads to expansion of infarct size and worsening of clinical outcome. The area undergoing secondary injury that surrounds the core of the ischemic lesion is termed the penumbra and is clinically attractive due to the delayed onset of pathogenic mechanisms, which provides a window of opportunity to salvage the penumbra via therapeutic intervention (5).

Neurons are energetically demanding yet have little energy reserves; therefore neurons rely heavily on properly functioning mitochondria for normal neuronal communication and activity. In addition to synthesizing ATP, the mitochondrion is also important in cell metabolism, calcium homeostasis, free radical production, and apoptosis (6-8). During the secondary phase of ischemic injury, these mitochondria-dependent pathways are disrupted leading to increased reactive oxygen species, intracellular calcium and induction of pro-apoptotic cascades (4, 9, 10). Thus, the development of pharmacological agents to promote recovery of mitochondria and ATP-

dependent cellular functions may limit secondary neuronal damage following cerebral ischemia.

Our laboratory previously demonstrated that decreases in the master regulator of MB, peroxisome proliferator-activated-receptor γ coactivator-1α (PGC-1α) is associated with decreased ability to maintain adequate mitochondria number in response to injury (11, 12). Mitochondria abundance and the integrity of mitochondrial DNA (mtDNA) is crucial for recovery of cellular function following ischemic injury. Therefore, the aim of this study was to examine the components of the PGC-1α signaling pathway including genes that encode for respiratory chain subunits and antioxidant genes, to understand the underlying mechanism that leads to alterations in mitochondrial homeostasis in an endothelin-1 (ET-1) induced experimental model of ischemic stroke to the caudal forelimb area of the sensorimotor cortex (SMC) (13). Furthermore, we examined two markers of inflammation in this model and related changes in these markers to the mitochondrial changes.

MATERIALS AND METHODS

Animals

Long Evans male rats (n=86, 3-4 months old) received food and water ad libitum and were kept on a 12:12hr light:dark cycle. Rats were randomly assigned to one of six groups that received either a sham or stroke procedure and were euthanized at one of three time points: 24hr (sham=10; stroke=10), 72hr (sham=14; stroke=22), or 144hr (sham=10; stroke=20). All animal protocols followed the National Institutes of Health (NIH)Guide for the Care and Use of Laboratory Animals, and were approved by the Medical University of South Carolina Animal Care and Use Committee.

Surgical Procedures

Rats were anesthetized with ketamine (1.1mg/kg I.P.) and Xylazine (0.7mg/kg I.P.). Unilateral ischemic lesions were induced via ET-1 (American Peptide, Inc) into a randomly assigned hemisphere in the forelimb area of the SMC (Fl-SMC). A craniotomy was performed at 1.0 mm posterior and 2.0 mm anterior to bregma and 3.0–5.0 mm lateral to midline and dura was gently retracted. ET-1 was applied on the brain surface at approximately 1ul/min, with a 2 min wait between applications using a total of 4ul. After the final 1ul of ET-1, the brain was left undisturbed for 5 min and then the craniotomy was covered with gel film (Invotec International) and dental acrylic. Sham animals had all procedures up to craniotomy. All animals received buprenorphine (0.5mg/kg S.C.) prior to incision for pain.

Ladder Task

To assess ischemia-induced impairments of forelimb function and compare these to mitochondria homeostasis markers, all animals were tested on the ladder task on days 0, 1, 3, and 6. The ladder task was used to assess coordinated forelimb use, stepping

accuracy, and limb placement and is sensitive to motor cortex damage (14).The ladder apparatus is made of two plexiglass walls, with 3mm diameter pegs spaced 1cm apart from each other. The ladder is raised ~20cm off the ground with a neutral start cage and the animal's home cage at the end. Through slow-motion video replay, all forelimb placements were qualitatively scored on a 0-6 rating scale over three trials (three traverses across the ladder). A perfectly placed limb received a score of 6. Errors were scored as follows: 0 = limb missed the ladder rung and the limb fell through the rungs; 1 = the limb was placed the limb but when weight bearing either fell (score of 1) or slipped (score of 2) (14). Percent errors was calculated as: sum of errors (0+1+2)/(total steps) per test day.

Tissue Collection

Animals were deeply anesthetized with Euthasol (0.1mg/kg) and brains were removed to obtain fresh tissue punches from the ipsilesional remaining sensory and motor cortex and the striatum. Samples were taken medial and anterior to the injury based on specific lesions and anatomical observation. Tissue was placed on dry ice to preserve mRNA and protein levels. Samples remained in a -80 freezer until RNA isolation or western blot analysis was performed.

RNA Isolation and Real-Time PCR

Total RNA was extracted from cortex and striatum using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Reverse transcription was performed using the RevertAid First Strand cDNA kit (Thermo Fisher Scientific) with 0.5-1 ug of RNA. 5 uL of cDNA template was used to amplify PCR products using 2x Maxima SYBR green qPCR master mix (Thermo Fisher Scientific). The primer sequences used in the qPCR protocol are listed in Table 5.1. Fold changes in mRNA expression were normalized to tubulin

were calculated using the ΔΔ- Ct analysis method detailed previously by Wills et. al. (15).

Mitochondrial DNA Content

Relative mtDNA content in rat cortex and striatum samples was measured using real-time PCR. DNA was isolated from tissues using the DNEasy Blood and Tissue Kit (Qiagen), 5 ng of cellular DNA was used to perform qPCR. Relative quantity of mtDNA was assessed by expression of NADH dehydrogenase 1 (ND1), a mitochondrial gene, and normalized to nuclear-encoded β-actin. Primer sequences for ND1 and *β*-actin were ND1 sense: 5'-TGAATCCGAGCATCCTACC-3′; ND1 antisense: 5'-ATTCCTGCTAGGAAAATTGG-3'; *β*-actin sense: 5'-TAAGGAACAACCCAGCATCC-3'; and *β*-actin antisense: 5'-CAGTGAGGCCAGGATAGAGC-3′. The ΔΔ-Ct analysis method was used to calculate fold changes in expression (15).

Immunoblot Analysis

Rat cortex and striatum tissue was homogenized in 150 µL of protein lysis buffer and protease inhibitors (1% Triton X-100, 150 mM NaCl, 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; 1 µg/ml leupeptin; and 1 µg/ml aprotinin) using a Polytron homogenizer. Then the samples were sonicated and centrifuged at 14,000 g for 15 min at 4°C. The supernatant was collected and protein quantified using a bicinchoninic acid kit (Sigma). Proteins (30 µg) were separated on 4 to 20% gradient SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were blocked in 5% BSA or milk in TBST (0.1% Tween 20 in 1× Tris-buffered saline) and incubated with primary antibodies overnight at 4°C. Primary antibodies used in this study included: COX1 (1:2000 Abcam); ND1 (1:2000, Abcam); NDUFS1 (1:2000, Abcam); cleaved caspase 3 (1:1000, Cell Signaling, Danvers); Caspase 3 (1:1000, Santa Cruz); GAP-43 (1:1000,

Cell Signaling) and GAPDH (1:10000, Fitzgerald). After incubation for 1hr at room temperature with secondary rabbit (1:2000) or mouse (1:20000) antibodies conjugated with horseradish peroxidase, membranes were detected by chemiluminescence. Densitometric analysis was performed using ImageJ (16).

Statistical Analysis

All data are reported as group means with ±S.E.M. Repeated analysis of variance (rANOVA) was used to test for behavioral differences with post-hoc comparisons for each post-operative day. Single comparison of molecular data was performed using the Student t-test, whereas data found to not have a normal distribution were subjected to a Mann-Whitney U-test. Sample size was determine based on a power analysis for AVOVA with effect size equal to 0.25, $\alpha = 0.05$, $\beta = 0.8$ and previous behavioral studies. Data were considered statistically significantly different at $p \leq 0.05$.

Table 5.1: Primer sequences used for RT-qPCR

Gene	Primer Sequence	
PGC-1α	Sense	5'-AGGAAATCCGAGCGGAGCTGA-3'
	Antisense	5'-GCAAGAAGGCGACACATCGAA-3'
NDUFS1	Sense	5'-AGATGATTTGGGAACAACGG-3'
	Antisense	5'-TAAGGCTTAGAGGTTAGGGC-3'
COX1	Sense	5'-CCTGAGCAGGAATAGTAGGG-3'
	Antisense	5'-AGTGGTACAAGTCAGTTCCC-3'
ND1	Sense	5'-TGAATCCGAGCATCCTACC-3'
	Antisense	5'-ATTCCTGCTAGGAAAATTGG-3'
SOD2	Sense	5'-CAAGGGAGATGTTACAACTCAGG-3'
	Antisense	5'-CTTAGGGCTCAGGTTTGTCCA-3'
UCP2	Sense	5'-GAGATACCAGAGCACTGTCG-3'
	Antisense	5'-GCTCAGTACAGTTGACAATGG-3'
F4/80	Sense	5'-TCCTCTTCTGGGGCTTCAGT-3'
	Antisense	5'-CCATTGCTGGGCAGAAAACC-3'
IL-6	Sense	5'-TTCAGAGCAATACTGAAACCC-3'
	Antisense	5'-GATGGTCTTGGTCCTTAGCC-3'
Tubulin	Sense	5'-CTCTCTGTCGACTACGGAAAG-3'
	Antisense	5'-TGGTGAGGATGGAATTGTAGG-3'
Actin	Sense	5'-TAAGGAACAACCCAGCATCC-3'
	Antisense	5'-CAGTGAGGCCAGGATAGAGC-3'

RESULTS

Stroke-induced Motor Impairment

As demonstrated previously, unilateral ET-1 lesions to the (Fl-SMC) result in lasting impairments in the forelimb opposite the lesion (14). Animals exhibited more limb placement errors while walking across a horizontal ladder with their impaired limb at 24, 72, and 144hr post-injury compared to sham animals (Fig. 5.1A). There were no differences seen with the non-impaired forelimb compared to sham animals or pre-stroke number of errors (Fig. 5.1B).

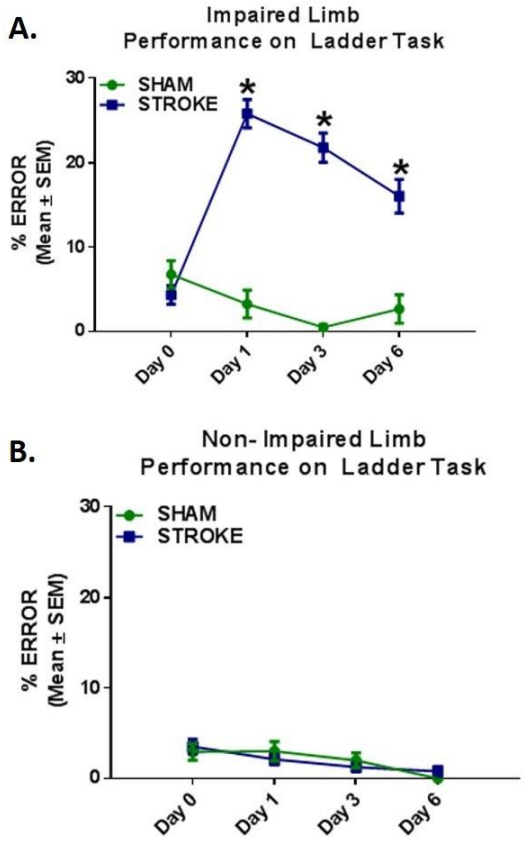

Figure 5.1: Stroke-induced motor impairment. Following injury, animals made significantly more errors with their impaired limb compared to sham controls (A). There was no difference in errors made with the non-impaired forelimb between stroke and sham animals (B). Data are reported as mean ± SEM, n = 6. *p<0.05 versus Sham animals.

Decreased PGC-1α and respiratory chain gene expression and mtDNA content in ipsilesional motor and sensory cortex

Mitochondrial dysfunction is a major contributor to neuronal death following ischemic stroke (7). Therefore, we assessed mRNA expression of PGC-1α, and components of the electron transport chain, nuclear-encoded NADH dehydrogenase (ubiquinone) Fe-S protein 1 (NDUFS1), and mitochondrial-encoded cytochrome c oxidase subunit 1 (COX1) and ND1. We observed a non-significant downward trend ($p= 0.056$) in PGC-1α mRNA expression 24hr post-stroke (Fig. 5.2A). NDUFS1 mRNA expression was decreased by 25% at 24hr and did not return to sham levels until 144hr post-stroke (Fig. 5.2B). Additionally, there was a robust reduction of COX1 and ND1 transcript levels at 72hr and remained suppressed by 50% at 144hr following injury (Fig.5.2C, D). Mitochondrial DNA copy number was assessed as a marker of mitochondrial content. There was a persistent suppression of mtDNA copy number to 74%, 71%, and 64% (24, 72, 144hr post-stroke, respectively) (Fig. 5.2E). Taken together, these findings reveal disruption in transcriptional regulation of mitochondrial proteins involved in oxidative phosphorylation and mitochondrial content following ET-1 induction of cerebral ischemia.

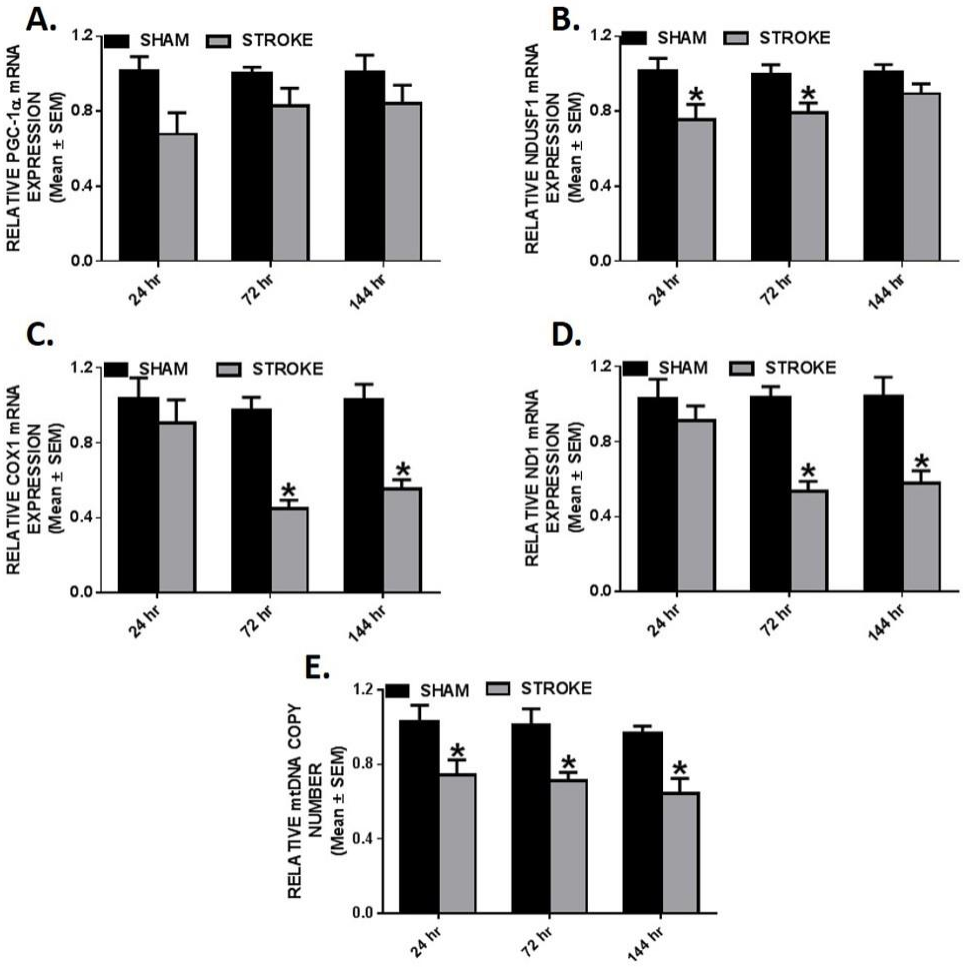

Figure 5.2: Decreased respiratory chain gene expression and mitochondrial DNA in ipsilateral cortex. Rats were subjected to either sham or ET-1 treatment. PGC-1α (A), NDUFS1 (B), COX1(C) and ND1 (D) mRNA expression was determined by RT-PCR using tubulin as a control gene. mtDNA copy number (E) was determined by qPCR, using ND1 for the mtDNA gene and actin for the nuclear control gene. These markers were measured in the ipsilateral cortex 24, 72, and 144 hr. Data are reported as mean ± SEM, n = 6. *p<0.05 versus Sham animals.

Transitory changes of mitochondrial encoded transcripts in ipsilesional striatum

We screened for mitochondria damage in the ipsilesional striatum following ET-1 ischemic damage due to secondary injury in this area (17). There were no changes in PGC-1α and NDUFS1 mRNA expression during the 24-144hr injury phase (Fig. 5.3A, B). However, we did observe a transitory decrease in COX1 and ND1 mRNA expression at 72hr post-stroke (70% and 53%, respectively). By 144hr, COX1 and ND1 mRNA levels recovered back to sham levels (Fig. 5.3 C, D). At 24hr, we detected a 25% reduction of mitochondrial DNA copy number in the ipsilesional striatum which returned to sham levels at 72hr (Fig. 5.3E). These results reveal that ET-1 induced SMC lesions have transitory damaging effects on the striatum, indicating that the striatum is susceptible to disruptions in mitochondrial homeostasis and function.

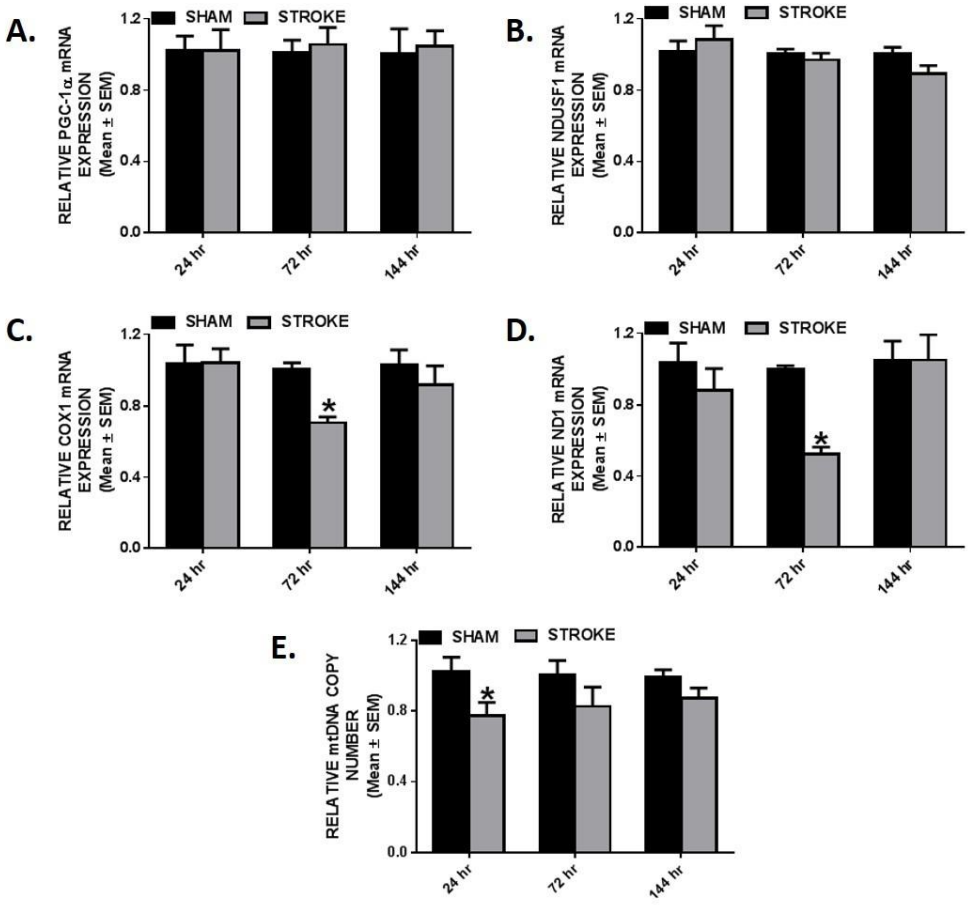

Figure 5.3: Altered regulation of mitochondrial- encoded transcripts in ipsilateral striatum. Rats were subjected to either sham or ET-1 treatment. PGC-1α (A), NDUFS1 (B), COX1(C) and ND1 (D) mRNA expression was determined by RT-PCR using tubulin as a control gene. mtDNA copy number (E) was determined by qPCR, using ND1 for the mtDNA gene and actin for the nuclear control gene. These markers were measured in the ipsilateral striatum 24, 72, and 144 hr. Data are reported as mean ± SEM, n = 6. *p<0.05 versus Sham animals.

Reduced mitochondrial encoded protein expression in the ipsilesional cortex and striatum

To assess if ET-1 disrupts the translation of mitochondrial genes we measured protein levels of COX1, ND1, and NDUFS1. Immunoblot analysis revealed that at 144hr, COX1 and ND1 were depressed to 67% and 76% compared to sham levels in the ipsilesional cortex (Fig. 5.4A). COX1 and ND1 protein expression were also decreased in the ipsilesional striatum to 80% and 77% (Fig. 5.4B). NDUFS1 remained at sham control levels at this 144hr time point. These data reveal that in our model of cerebral ischemia mitochondrial-encoded genes are more sensitive to transcriptional/translational disruption than nuclear-encoded genes.

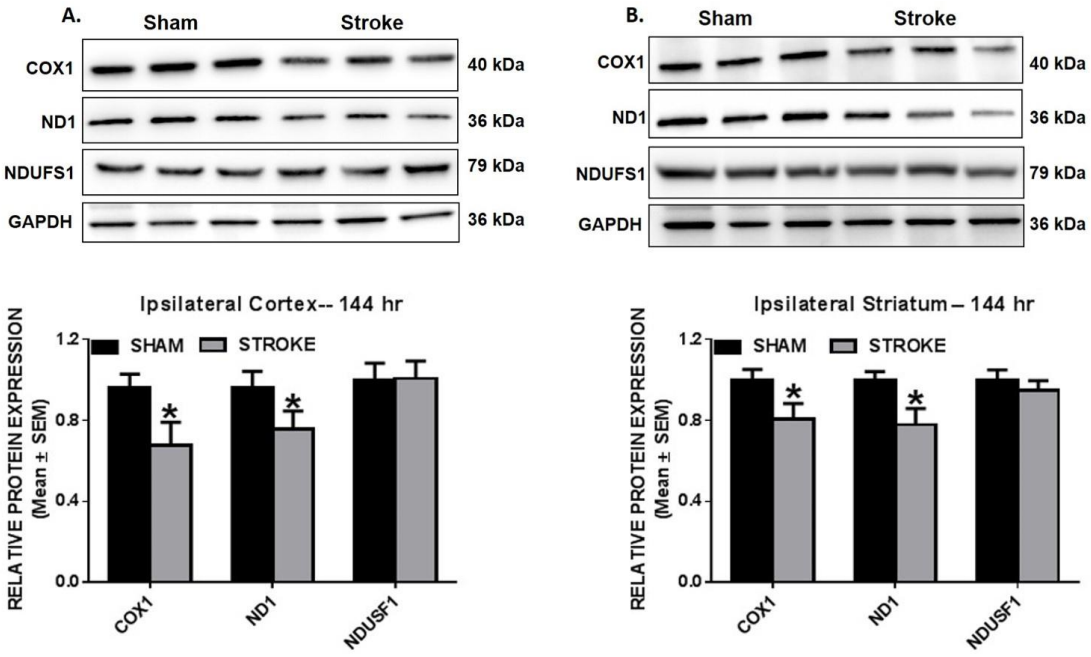

Figure 5.4: Altered respiratory chain protein expression in ipsilateral cortex and striatum. Rats were subjected to either sham or ET-1 treatment. COX1, ND1, and NDUFS1 protein expression was determined by immunoblot analysis. These markers were measured in protein isolated from ipsilateral cortex (A) and striatum (B) 144 hr following ET-1 exposure. These markers were measured in the ipsilateral striatum 24, 72, and 144 hr. Data are reported as mean ± SEM, n = 6. *p<0.05 versus Sham animals.

UCP2 activation in the ipsilesional cortex and striatum

Excessive reactive oxygen species (ROS) generation can induce the up regulation of mitochondrial ROS detoxifying enzymes, manganese superoxide dismutase 2 (SOD2) and the uncoupling protein 2 (UCP2) to neutralize the detrimental effects initiated by oxidative stress. Along with its uncoupling role to mitigate ROS production, UCP2 has been implicated in neuroprotection by suppressing pro- inflammatory cytokines and elevating anti- apoptotic mediator, Bcl2 (18). Therefore, we measured SOD2 and UCP2 in ipsilesional cortex and striatum. Interestingly, we only observed a modest 17% decrease of SOD2 transcript at 72hr in the cortex and SOD2 mRNA levels remained unchanged in the striatum (Fig. 5.5A, C). In contrast, cortical UCP2 transcript levels increased by ~1.5-fold within 24hr and further increased to ~3-fold by 72hr which persisted to 144hr (Fig. 5.5B).In the ipsilesional striatum, UCP2 increased 1.5-fold over sham control at 72hr and remained elevated at 144hr (Fig. 5.5D). These data reveal that ET-1 mediates sustained increases in UCP2 in cortex and striatum.

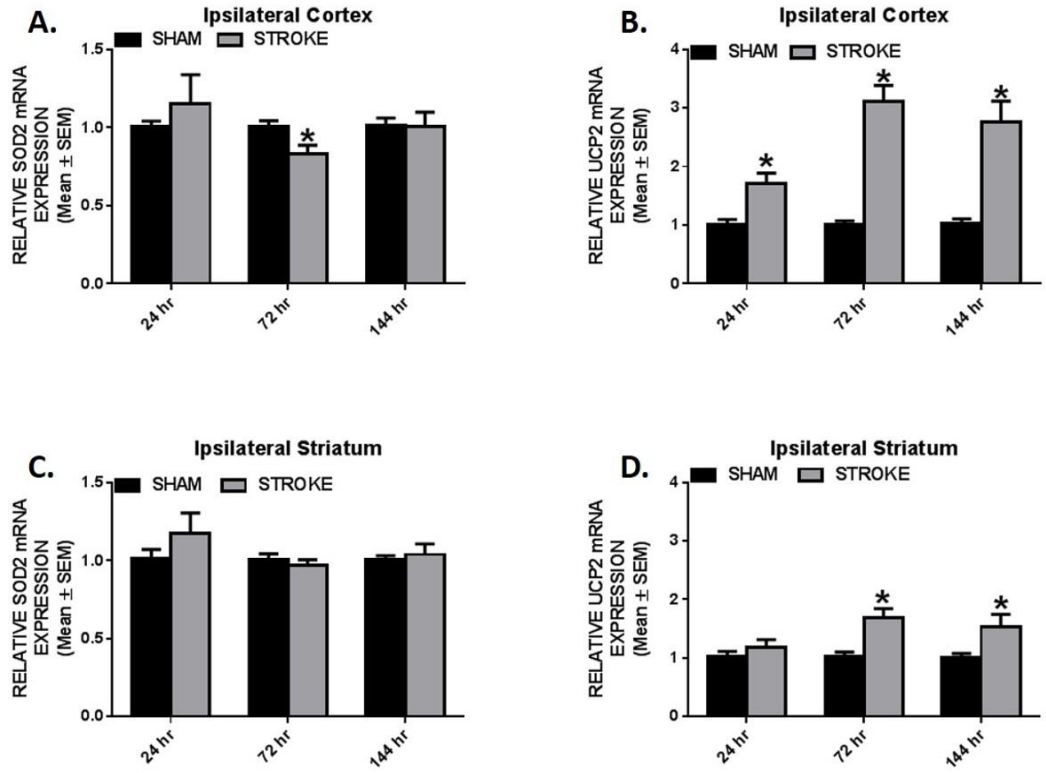

Figure 5.5: Activated antioxidant gene expression in ipsilateral cortex and striatum. Rats were subjected to either sham or ET-1 treatment. Cortical (A, B) and striatal (C, D) SOD2 and UCP2 mRNA expression was determined by RT-PCR using tubulin as a control gene. These markers were measured 24, 72, and 144 hr following ET-1 exposure. Data are reported as mean ± SEM, n = 6. *$p<0.05$ versus Sham animals.

Gene expression of inflammatory mediators and macrophages in the ipsilesional cortex and striatum

Since macrophages are known to be involved in innate immune response following ischemic injury (19), we examined the transcriptional expression of F4/80, a surface marker for mature macrophages. We observed a 5-fold increase in cortical F4/80 mRNA expression over sham animals at 72 and 144hr (Fig. 5.6A). Additionally, we observed significant 3-, 2- and 1.5-fold increases in cortical interleukin-6 (IL-6) transcripts at 24, 72, and 144hr, respectively (Fig. 5.6B). We detected a transitory 1.5-fold increase in F4/80 at 72hr that returned to sham levels by 144hr in the striatum (Fig. 5.6C). IL-6 induced a 2.5-fold increase at 24hr and reduced to 1.5-fold increase 72hr in the striatum (Fig. 5.6D). Our data provides evidence and a partial time-course for a pro-inflammatory environment in both the cortex and striatum following ischemic stroke.

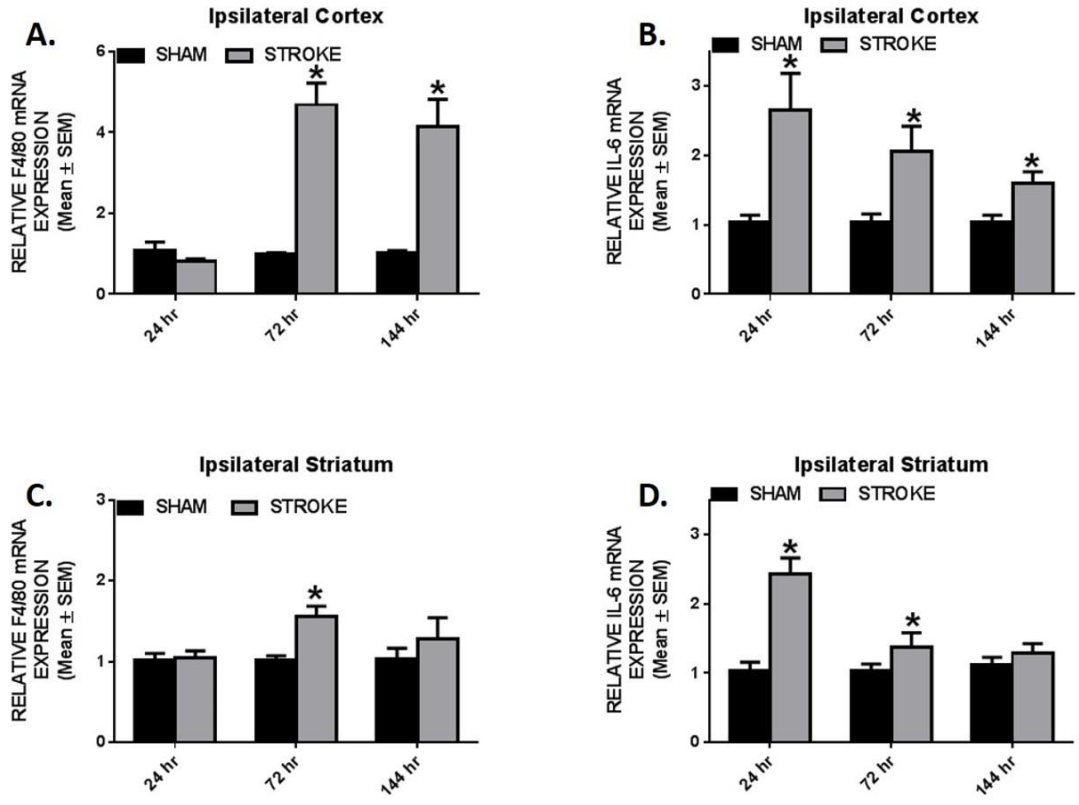

Figure 5.6: Induced gene expression of inflammatory mediators in ipsilateral cortex and striatum. Rats were subjected to either sham or ET-1 treatment. Cortical (A, B) and striatal (C, D) F4/80 and IL6 mRNA expression was determined by RT-PCR using tubulin as a control gene. These markers were measured 24, 72, and 144 hr following ET-1 exposure. Data are reported as mean ± SEM, n = 6. *$p<0.05$ versus Sham animals.

Caspase 3 cleavage and GAP43 expression in ipsilateral cortex and striatum

Rodent models of neurodegenerative diseases implicate caspase dependent apoptosis as an important contributor to neuronal and tissue damage (20-23) Immunoblot analysis was used to assess the level of caspase 3 activation by measuring the cleaved form of caspase 3 following ET-1 induced stroke. Cleaved caspase 3 was detected in the ipsilateral cortex, but not in the ipsilateral striatum 144hr following stroke (Fig.5.7A, B).

To determine growth factor expression in our ET-1 model of ischemic stroke we measured GAP-43, a growth factor known to increase during periods of axonal sprouting within the first week post- stroke in areas surrounding the primary site of injury (24, 25). In line with previous reports, we observed a 3-fold increase in GAP-43 protein expression in the ipsilesional cortex at 144hr (Fig. 5.7C). We also detected a 4-fold increase in the striatum at 144hr post–stroke (Fig. 5.7D). These data reveal that sufficient cell injury occurs in the early stages post-stroke to activate regenerative processes.

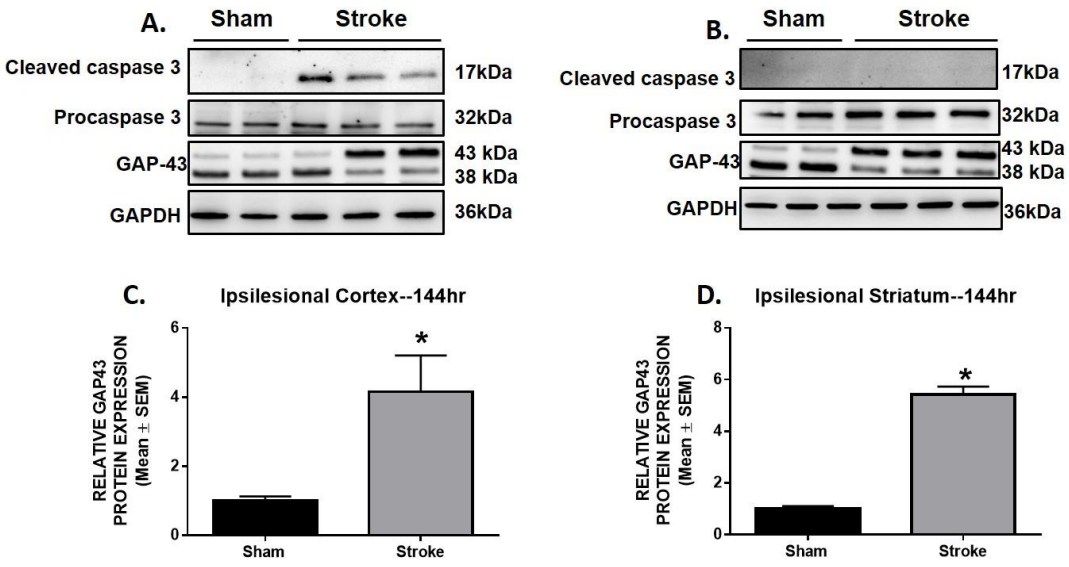

Figure 5.7: Caspase 3 cleavage and GAP43 expression in ipsilateral cortex and striatum. Rats were subjected to either sham or ET-1 surgeries. Cleaved caspase 3, procaspase 3, and GAP-43 protein expression was determined by immunoblot analysis. Protein was isolated from ipsilesional cortex (A) and striatum (B) 144hr post- injury. Cortical (C) and striatal (D). GAP-43 protein expression were quantified. GAPDH is used as loading control. Data are reported as mean ± SEM, n = 6. *p<0.05 versus Sham animals.

DISCUSSION

Several studies have demonstrated mitochondrial dysfunction as a common consequence of cerebral ischemia including mitochondrial induced-oxidative stress (9, 26, 27). ROS signals for both beneficial and detrimental pathways following injury. Beneficial ROS signaling initiates neuroprotective pathways such as ROS-scavenging mechanisms (28). However, excessive ROS causes oxidative damage to a multitude of biomolecules such as mtDNA. Additionally, elevated ROS levels promote the release of inflammatory cytokines and subsequent migration of inflammatory cells to the ischemic and penumbra zones promoting an inflammatory environment that can mitigate injury progression or boost the progression of neuronal damage (19, 29).

Using an ET-1-induced rat stroke model, we identified mitochondrial disruption and activation of inflammatory responses in the SMC and striatum during the first week post-injury. In the cortex, we observed decreased expression of nuclear and mitochondrial encoded subunits of complex I and IV, and depletion of mitochondrial DNA number. Specifically, NDUFS1, complex I gene expression is suppressed at the early 24hr time point and does not return to sham control levels until 144hr post- stroke. A robust decrease in both COX1 and ND1 transcript levels, subunits of complex IV and I, respectively, was observed at 72hr and 144hr. Importantly, we also detected significantly reduced COX1 and ND1 protein expression at the 144hr time point. Concomitantly, mitochondrial DNA copy number was suppressed early and persistently in the ipsilesional cortex. We only observed reduced protein expression in mitochondrial-encoded proteins which may be due to the increased sensitivity of mtDNA damage compared to nuclear DNA. Following ischemic injury, chronic ROS production causes extensive mtDNA damage. The inability to monitor and repair mtDNA damage is due to the less efficient mitochondrial repair mechanisms that become overwhelmed in the

presence of excessive oxidative stress (30).It is expected that mtDNA damage may lead to inactive the electron transport chain thereby affecting normal mitochondrial function.

We also detected disruption of mitochondrial homeostasis in the ipsilateral striatum. Based on the functional connectivity between the cortex and striatum, we propose that reduction of mitochondrial DNA and mRNA expression of ND1 and COX1 may be secondary consequences. Additionally, our experimental cerebral ischemia model disrupted the translation of COX1 and ND1 at 144hr post-stroke. The presence of mitochondrial suppression in the cortex and striatum depicts how pathogenic mechanisms can affect adjacent cells, intensifying the damaging effects of ischemic stroke. It is important to note that although we did not report a significant decrease in PGC-1α mRNA expression in either the cortex or striatum, we do hypothesize that the PGC-1α transcript levels are reduced abruptly following injury and recover towards control levels to regulate compensatory mechanisms such as antioxidant mechanisms. Under normal physiological conditions, mitochondrial respiratory complexes are a source of ROS generation (7). When respiratory enzymes are damaged following injury, these complexes produce an excessive amount of ROS which can lead to cytotoxicity. Therefore, we assessed mRNA level of the mitochondrial antioxidant gene, SOD2 as a marker of oxidative stress. We only observed a decrease in SOD2 transcript at 72hr post-stroke in the cortex, which recovered to sham control levels by 144hr and SOD2 mRNA levels remained unchanged in the striatum. Numerous studies have reported SOD2 is a direct downstream target of PGC-1α (31, 32); therefore, we predict that SOD2 mRNA levels transiently decreased in response to the modest suppression of PGC-1α in the cortex at 24hr.

Recently, an increasing number of studies have focused on the various physiological and pathological roles of UCP2 (33-36). When UCP2 detects elevated levels of

mitochondrial ROS, a feedback loop is activated to induce UCP2 expression in the inner mitochondrial membrane (36). It is well documented that the primary role of UCP2 is to dissipate the proton gradient across the inner membrane to prevent ATP synthesis and transporting superoxide radicals across the inner mitochondrial membrane, thus decreasing the ROS produced by the respiratory chain (34). Interestingly, UCP2 mRNA expression was persistently increased in both the ispilesional cortex and striatum. While cortical UCP2 induction occurred at 24hr, prior to the striatum, UCP2 expression remained elevated in both tissues.

Additionally, UCP2 has been reported to perform other functions such as regulating neuroinflammation and apoptosis following ischemic stroke. Haines, et al., demonstrated that the overexpression of UCP2 alleviated ischemia-induced increase in IL-6 mRNA which may reduce the deleterious effects of prolonged inflammation. Overexpression of UCP2 also rescued diminished pro-survival markers such as Bcl-2, cyclin G2, and HSP90 (18). Furthermore, UCP2 has been documented to be neuroprotective via its involvement in neurogenesis and synaptogenesis, suggesting a role in neuronal growth and development (36, 37). Future studies are needed to explore the potential roles of UCP2 in our cerebral ischemic model.

The presence of neuroinflammation during the first days of experimental stroke was confirmed by the elevated mRNA expression of macrophage marker, F4/80 and inflammatory mediator cytokine, IL-6. We observed a 5-fold and 4-fold induction of F4/80 in the cortex at 72 and 144hr, respectively. Interestingly, cortical IL-6 transcript levels were induced earlier with a magnitude of 3-fold and remained elevated above sham control levels. Furthermore, the maximal expression of F4/80 and IL-6 in the cortex corresponds to the maximal expression of both transcripts in the striatum. Taken together, these results support the implication that ischemic injury causes detrimental

effects in surrounding tissue days after the initial stroke; thus, the opportunity to effectively treat per-infarct tissue extends into the post-acute recovery period.

One of the most common forms of cell death in neurodegeneration is through the intrinsic mitochondrial apoptotic pathway. Following initiation of intrinsic pathway, cytochrome c is released from the mitochondria and works with other apoptotic factors to process the inactive form of procaspase 3 to the cleave, active form (38). Cleaved caspase 3 in turn induces cellular changes including chromatin condensation, DNA fragmentation, and formation of apoptotic bodies (39, 40). Our study revealed that cerebral ischemia induced caspase 3 cleavage in the peri-infarct region of ipsilateral cortex 144hr post-stroke. These data reveal that activation of cell injury and death pathways, which produces long term effects, occur early after cerebral ischemia, strengthening the importance for the characterization of molecular and metabolic events that lead to ischemic cell loss as well as repair and regenerative mechanisms that can function to preserve the function of surrounding neurons (41, 42).

Regenerative processes, such as axon sprouting and new synapse formation, following stroke begin within days and continues for weeks after injury. GAP-43 induces formation of new synapses, enhance axonal sprouting post injury, and may play an important role in experience-dependent plasticity (43). Knockout of GAP-43 results in a decrease in axon sprouting and hindered growth cone navigation (44, 45). Previous studies demonstrate that elevated GAP-43 expression occurs between days 3 and 14 suggesting growth cone development, whereas axonal sprouting and new synapse formation occurs between days 14 and 60 post-stroke (24, 25, 46, 47). Consistent with the above findings, GAP-43 increased in cortex and striatum at 144hr in our ET-1 model of ischemic stroke, demonstrating that sufficient cell injury occurs in the early stages

post-stroke to activate regenerative processes needed to preserve and restore neuronal function.

CONCLUSION

In conclusion, ET-1-induced focal experimental stroke to the SMC leads to mitochondrial dysregulation, and inflammatory cell infiltration during the first week of injury which last days following initial injury. We also observed alterations in these pathways in the striatum, a secondary site of damage and degeneration. This suggests that the striatum is highly susceptible to cellular injury associated with deficits in mitochondrial homeostasis. This finding, along with the suppression of mitochondrial proteins and sustained gene aberrations warrant further investigation to elucidate chronic changes in these pathways related to long-term behavior outcomes. Further examination may lead to the identification of therapeutic targets to treat ischemic stroke by reduction of mitochondrial dysfunction, bioenergetics failure, and inflammation.

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