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Elucidation of the Mechanism of Beta-2 Adrenergic Receptor Agonist-Mediated Mitochondrial Biogenesis

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Elucidation of the Mechanism of Beta-2 Adrenergic Receptor Agonist-Mediated Mitochondrial Biogenesis by

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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

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Dedication

I would like to dedicate my dissertation to my parents, Bob and Deb Cameron, and to my partner, Kristina Wright. Without your support and kind words, the last five years would have been much more difficult. Thank you.

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Symbols and Abbreviations

ATP, adenosine triphosphate;

AMP, adenosine monophosphate;

MPTP, mitochondrial permeability transition pore;

MB, mitochondrial biogenesis;

PGC-1α, peroxisomal proliferation activated receptor coactivator-1α;

mtDNA- mitochondrial DNA;

ER, estrogen receptor;

ERRα, estrogen related receptor-α;

NRF-1, nuclear respiratory factor 1;

NRF-2, nuclear respiratory factor 2;

PPAR, peroxisome proliferator-activated receptor;

TR, thyroid hormone;

CREB, cAMP-responsive element binding protein;

YY-1- yin yang-1;

ROS- reactive oxygen species;

PGC-1, peroxisomal proliferation activated receptor coactivator-1;

PGC-1β, peroxisomal proliferation activated receptor coactivator-1β;

PRC, PGC-1 related coactivator;

PKA, protein kinase A;

NO, nitric oxide;

AMPK, growth stimulatory AMP-dependent kinase;

SIRT1, silent mating type information regulation 2 homolog 1;

AKI, acute kidney injury;

Tfam, mitochondrial transcription factor A;

UCP2, uncoupling protein 2;

PINK1, PTEN-induced putative kinase 1;

NAD^+, nicotinamide adenine dinucleotide;

SOD2, superoxide dismutase 2;

SIRT3, silent mating type information regulation 2 homolog 3;

eNOS, endothelial nitric oxide synthase;

TZD, thiazolidinedione;

PPARγ, peroxisomal proliferation activated receptor-γ;

ERα, estrogen receptor α;

ERβ, estrogen receptor β;

GPER, G protein-coupled estrogen receptor;

ALDH2, aldehyde dehydrogenase 2;

LDL, low density lipoprotein;

ERK 1/2, extracellular signal-related kinases 1/2;

MEK 1/2, mitogen-activated protein kinase kinase 1/2;

sGC, soluble guanylate cyclase;

PDE, phosphodiesterase;

PKG, Protein kinase G;

VEGF, vascular endothelial growth factor;

HGF, hepatocyte growth factor;

HbA_{1c}, glycated hemoglobin;

GPCR, G protein-coupled receptor;

GRK, G protein-coupled receptor kinase;

GIT1, GRK interacting protein 1;

CB1R, cannabinoid-1 receptor;

5-HT, 5-hydroxytryptamine;

TBHP, *tert*-butyl hydrogen peroxide.

AKI; acute kidney injury

β_2AR; beta-2 adrenergic receptor

Drp1; dynamin-related protein 1

FCCP; carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone

IBMX; 3-isobutyl-1-methylxanthine

IR; ischemia-reperfusion

Mfn2; mitofusin 2

NOS; nitric oxide synthase

PI3K; phosphatidylinositol-4,5-biphosphate 3-kinase

RPTC; renal proximal tubule cell

ESRD; end-stage renal disease

KDIGO; Kidney Disease Improving Global Outcomes

CKD; chronic kidney disease

eGFR; estimated glomerular filtration rate

ATN; acute tubular necrosis

ARDS; acute respiratory distress syndrome

eNAC; epithelial sodium channel

SCr; serum creatinine

NGAL; neutrophil gelatinase-associated lipocalin

L-FABP; liver-type fatty acid binding protein

IL-18; interleukin 18

IGFBP7; Insulin-like growth factor binding protein 7

TIMP-2; tissue inhibitor of metalloproteinase-2

DAMP; damage-associated molecular pattern

KIM-1; kidney injury marker-1

NFκB; nuclear factor kappa-light-chain-enhancer of activated B-cells

TNF-α; tumor necrosis factor α

IL-6; interleukin 6

IL-8; interleukin 8

MCP-1; monocyte chemoattractant protein-1

HIF-1α; hypoxia inducible factor-1 alpha

IFN-γ; interferon gamma

IL-17; interleukin 17

CSF-1; colony stimulating factor 1

IRAK; interleukin-1 receptor-associated kinse 4

NK cell; natural killer cell

IL-4; interleukin 4

IL-10; interleukin 10

T_{REG} cell; regulatory T cell

CTLA-4; cytotoxic T-lymphocyte associated protein 4

IL-16; interleukin 17

STAT; signal transducer and activator of transcription

TGF-β; transforming growth factor beta

BNIP3; BCL2 interacting protein 3

FUNDC1; FUN14 containing 1

LC3; microtubule-associated protein 1A/1B-light chain 3

mTOR; mammalian target of rapamycin

MAP kinase; mitogen-activated protein kinase

TM; transmembrane domain

ECL; extracellular loop

SEM; standard error of the mean

FCCP-OCR; FCCP-uncoupled oxygen consumption rate

L-NAME; N(G)-nitro-*L*-arginine methyl ester

ODQ; 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one

NDUFS1; NADH-ubiquinone oxidoreductase core subunit S1

COX1; cytochrome c oxidase subunit 1

LPS; lipopolysaccharide

NMR; nuclear magnetic resonance

ND1; NADH dehydrogenase subunit 1

ND6; NADH dehydrogenase subunit 6

WT; $ADRB2^{Flox/Flox}$

KO; γGT-Cre:$ADRB2^{Flox/Flox}$

Abstract

Acute kidney injury (AKI) is the transient loss of renal function following an insult. Despite high incidence and mortality, therapy for AKI is limited to supportive care and renal replacement therapy. The induction of mitochondrial biogenesis (MB) accelerates recovery of renal function in animal models of AKI. We have identified that the beta-2 adrenergic receptor (β_2AR) agonist formoterol induces MB in renal proximal tubule cells (RPTC); however, not all β_2AR agonists induce MB. We hypothesized that formoterol activates a distinct signaling pathway in RPTC to induce MB and accelerate recovery of renal function. In cultured RPTC, we found that formoterol activates a Gβγ-Akt-eNOS-cGMP pathway. Clenbuterol, a β_2AR agonist that does not induce MB, did not activate this pathway. Formoterol, but not clenbuterol, increased mRNA expression of PGC-1a and NDUFS1; mtDNA copy number; and FCCP-uncoupled respiration at 24 h in RPTC. Inhibition of Gβγ, Akt, NOS, and guanylate cyclase prevented formoterol-induced increases in these markers of MB. To assess the role of proximal tubule β_2AR in formoterol-induced recovery of renal function, a mouse with proximal tubule-specific deletion of the β_2AR (γGT-Cre:ADRB2$^{Flox/Flox}$) was generated. Following bilateral renal ischemia reperfusion, γGT-Cre:ADRB2$^{Flox/Flox}$ and wild-type controls (ADRB2$^{Flox/Flox}$) were treated once-daily with 0.3 mg/kg formoterol beginning at 24 h. At 144 h post-injury, ADRB2$^{Flox/Flox}$ mice treated with formoterol had improved renal function and increased markers of MB relative to vehicle controls, while γGT-Cre:ADRB2$^{Flox/Flox}$ mice treated with formoterol did not. Furthermore, transmission electron microscopy demonstrated that in ADRB2$^{Flox/Flox}$ mice, but not in γGT-Cre:ADRB2$^{Flox/Flox}$ mice, formoterol increased mitochondrial number and density relative to vehicle controls. Together, these data demonstrate that formoterol activates a Gβγ-Akt-eNOS-cGMP to induce MB in RPTC and that following AKI, formoterol acts on RPTC β_2AR to induce MB and accelerate recovery of renal function.

CHAPTER 1

INTRODUCTION

Acute Kidney Injury

Acute kidney injury (AKI) is the rapid and usually reversible loss of renal function. The decline in renal function leads to the dysregulation of electrolytes and other metabolites, and it increases the likelihood of future bouts of AKI and the development of chronic kidney disease (CKD) and end-stage renal disease (ESRD). Current clinical standards as established by the 2012 Kidney Disease: Improving Global Outcomes (KDIGO) guidelines define AKI as: an increase in serum creatinine of $\geq$0.3 mg/dL within 48 h, an increase in serum creatinine to $\geq$1.5 times the baseline within the last seven days, or a urine output of <0.5 mL/kg/h for 6 hours. Under these standards, patients with AKI are classified into the following stages:

> Stage 1: increase in serum creatinine to 1.5-1.9 times the baseline, OR increase in serum creatinine by $\geq$0.3 mg/dL, OR reduction in urine output to <0.5 mL/kg for 6-12 h.
>
> Stage 2: increase in serum creatinine to 2.0-2.9 times the baseline, OR reduction in urine output to <0.5 mL/kg/h for $\geq$12 h.
>
> Stage 3: increase in serum creatinine to 3.0 times baseline, OR increase in serum creatinine to $\geq$4.0 mg/DL, OR reduction in urine output to <0.3 mL/kg/h for $\geq$24 h, OR anuria for $\geq$12 h, OR the initiation of renal replacement therapy, OR a decrease in estimated glomerular filtration rate (eGFR) to <35 mL/min/1.73 m^2 in pediatric patients.[1]

AKI affects over 20% of hospitalized patients and over 50% of patients in intensive care settings and carries a mortality rate of greater than 20%.[2,3] The causes of AKI are classified as prerenal, intrinsic, or postrenal.[4-6] Prerenal AKI is caused by drastic decreases in renal perfusion such as in diarrhea, acute hemorrhage, or hypovolemic states (e.g., heart failure, liver disease). Prerenal disease may also be caused by pharmacologic agents that interfere with renal vascular tone, such as nonsteroidal anti-inflammatory drugs (NSAIDS), ACE inhibitors, or radiopaque contrast. Intrinsic AKI is caused by direct damage to the renal vasculature, glomeruli, tubules, or interstitium. The most common cause of intrinsic AKI, and of AKI in general is acute tubular

necrosis (ATN) following ischemia, sepsis, or nephrotoxicant exposure.[6] Finally, postrenal AKI is caused by obstruction along the urinary tract. In healthy patients, the obstruction usually impacts all functioning kidneys, such as in prostatic hyperplasia or cancer, metastatic cancer, or retroperitoneal fibrosis involving the ureters. As both prerenal and postrenal causes of AKI can lead to ATN if untreated, many patients have multiple causes for developing AKI, thereby complicating treatment. Additionally, the current biomarkers of serum creatinine and urine output do not change until significant losses in renal function have already occurred,[7] thereby complicating the identification of relevant renal insults.

Most patients with AKI exhibit a full recovery of renal function within 21 days as measured by serum creatinine and urine output. A recent study also identified three subtypes of recovery from AKI:

1. An early (within seven days of diagnosis) and sustained recovery;
2. An early recovery followed by relapse and subsequent recovery; and
3. A late (greater than seven days after diagnosis) recovery.[8]

Sustained recovery was the most common and had the best prognosis, while a late recovery was the least common but still had improved survival at 12 months compared with patients who did not recover from AKI or underwent a relapse from which they did not recover. Furthermore, patients with prior incidents of AKI had a greater likelihood of developing CKD or ESRD,[9-13] indicating that there was some persistent renal dysfunction following AKI. Interestingly, the major causes of in-hospital mortality are often infection or the underlying condition that causes AKI.[14]

Due to their renal dysfunction, patients with AKI may exhibit fluid retention and ion imbalances, particularly hyperkalemia or hyponatremia; however, many patients develop no symptoms and their diagnosis is only identified by routine laboratory tests for serum creatinine.[15] Despite the initially silent presentation, AKI leads to dysfunction in multiple other organ systems.

The most commonly described distant manifestation of AKI is cardiorenal syndrome, with the term "cardiac failure" being used to describe cause of death in AKI patients.[16] There is an established relationship between AKI and the development of heart failure and arrhythmias as well as other forms of cardiovascular disease such as hypertension.[17,18] These outcomes may be due to fluid overload, endothelial cell activation, and inflammatory cytokines leading to cardiac cell death.[19] Animal models of AKI have shown cardiac dysfunction characterized by left ventricular dilation, increased relaxation time, and decreased fractional shortening.[20]

In addition to the cardiac effects of AKI, dysfunction of several other organ systems has been correlated with AKI. Patients with pulmonary dysfunction such as acute respiratory distress syndrome (ARDS) had a greater incidence of AKI and greater mortality following AKI.[21-23] Many patients who develop AKI also developed respiratory failure and associated increases in the inflammatory cytokines IL-6 and IL-8.[23-27] Animal models of AKI have demonstrated increased pulmonary vascular permeability and fluid accumulation following AKI due to decreased expression of epithelial sodium channels (eNACs) and aquaporins.[28-30] Different causes of AKI (e.g., sepsis, ischemia reperfusion, nephrectomy) in animal models also had distinct profiles of inflammatory cytokines in the lungs.[29,31] In the central nervous system, uremic encephalopathy and other forms of altered mental status have been associated with AKI,[32,33] but the pathogenesis is not well understood in humans. In the brains of animals subjected to AKI, there were alterations in calcium and water handling and dopamine turnover along with disruption of the blood brain barrier and increases in inflammation.[34-37] As a result, these animals exhibited diminished locomotor function. Despite these changes, there were no observed increases in apoptosis in the brain.

Because AKI has such profound effects on patient health, the underlying processes that lead to injury must be understood to improve the prevention, detection, and therapy of AKI. As acute tubular necrosis is associated with such a large proportion of AKI cases, much of the work

understanding the pathophysiology of AKI has focused on the proximal tubule. Proximal tubule injury has been shown to be sufficient for the development of AKI and, perhaps more importantly, the transition to CKD.

Since AKI leads to severe and systemic complications, the early identification of AKI is important.[38] Clinically, the standard biomarkers are serum creatinine (SCr) as a measure of eGFR and urine output. However, as creatinine is affected by muscle mass,[39] an increase in SCr may be masked by muscle wasting; this may be found in elderly patients or patients undergoing cancer chemotherapy. SCr is also relatively insensitive to decreases in GFR.[40] While serial measurements of creatinine can help improve diagnosis of AKI, serial measurements are not always available. Both SC and urine output are also measures of renal function rather than of renal injury and are therefore unable to distinguish among different etiologies of AKI.[41] As a result, neither of these metrics alone is necessarily able to predict mortality or disease severity.[42,43] To facilitate the early diagnosis of AKI and better guide treatment decisions, more sensitive and more specific biomarkers are needed (Figure 1-1).

Neutrophil gelatinase-associated lipocalin (NGAL) is expressed by neutrophils and binds to iron-siderophore complexes to prevent bacterial growth.[44,45] Following ischemia- or nephrotoxicant-induced AKI, NGAL expression is rapidly upregulated,[46,47] with elevated levels appearing at 3 h and persisting for up to 5 days in cases of severe injury. Within the kidney, NGAL is mainly produced by the thick ascending loop of Henle and the intercalated cells of the collecting duct.[48,49] NGAL is freely filtered by the glomerulus and is reabsorbed in the proximal tubules.[50] Once reabsorbed, NGAL upregulates the renoprotective enzyme heme-oxygenase 1.[51] Clinically, NGAL elevations preceded SCr increases, and NGAL is only elevated in the setting of damage to the kidney itself (i.e., intrinsic AKI, particularly that involving proximal tubule injury) rather than more rapidly reversible prerenal AKI.[52-54] The elevation of NGAL matched the

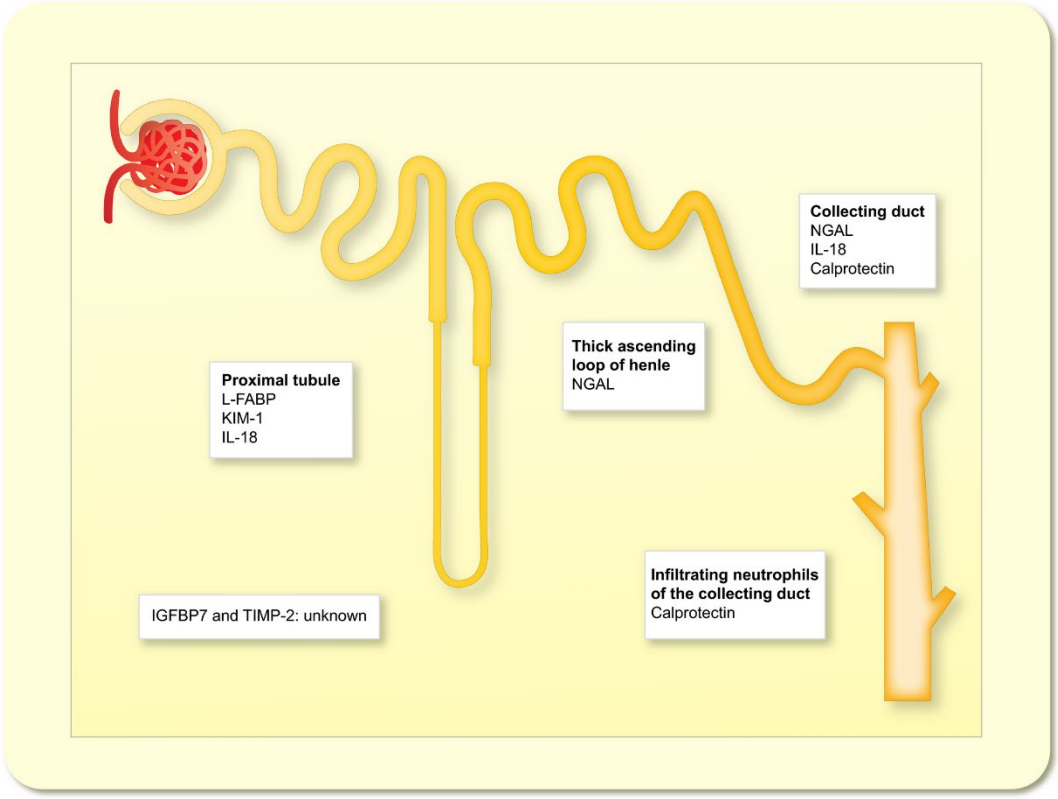


Figure 1-1. Sites of synthesis of novel biomarkers for the diagnosis of AKI. The many cell types of the nephron increase the expression of different markers following AKI. These biomarkers can be used to diagnose AKI, determine the causal insult, and monitor responses to therapy in human patients and animal models. Adapted from Schrezenmeier E.V., et. al. Biomarkers in Acute Kidney Injury. *Acta Physiol* 2016.

duration of AKI and predicts disease severity in terms of death or need for renal replacement therapy. However, because NGAL is expressed by neutrophils, it is also associated with sepsis,[55] chronic kidney disease,[56] and urinary tract infections,[40,49] so other biomarkers are required to confirm the presence of AKI.

Liver-type fatty acid binding protein (L-FABP) binds to fatty acids and transports them to mitochondria and peroxisomes for metabolism.[57] It also plays a role in antioxidant defense against hydrogen peroxide. In the kidney, L-FABP is primarily expressed in proximal tubules, where it is excreted bound to toxic peroxisomal byproducts.[58,59] Expressing L-FABP in mice reduced injury and preserved renal function following renal ischemia;[59,60] however, since mice do not normally express L-FABP, human-L-FABP transgenic mice are required to study the effects of L-FABP preclinically.[60,61] Clinically, L-FABP predicted the development of AKI in an intensive care unit (ICU) setting following surgery,[62] sepsis,[63] or nephrotoxicant exposure.[64] However, there are limited data regarding the predictive value of L-FABP for mortality or end-stage renal disease.[38]

Inteleukin-18 (IL-18) is a cytokine secreted by monocytes/macrophages during inflammation to induce the production of IFN-γ.[65] Following toll-like receptor (TLR) activation, other inflammatory signals, or injury, tubular cells produce IL-18.[66] Deficient IL-18 production or inhibition of its signaling was protective against AKI in rodent models.[67-71] As a biomarker, IL-18 had predictive value for AKI following kidney transplant and in pediatric patients but not in the ICU or ER settings.[53,72-75] More importantly, IL-18 may have value for guiding anti-IL-18 treatment decisions for patients with AKI.[38]

Cell cycle arrest in the G1 phase has been demonstrated following ischemic or septic AKI and is protective in animal models of nephrotoxic AKI.[76-79] IGF-binding protein 7 (IGFBP7) and tissue inhibitor of metalloproteinase-2 (TIMP-2) induce cell cycle arrest in multiple cell types, including breast and colon cancer and microvascular cells.[80-82] Additionally, urinary

concentrations of both TIMP-2 and IGFBP7 were elevated following AKI in rodents and humans.[83,84] However, there are no experimental data for their involvement in the renal cell cycle arrest, and the source of these proteins following AKI is unclear.[38] Clinically, the product of the concentrations of IGFBP7 and TIMP-2 was a good predictor of severe (stage 2 or 3) AKI,[85,86] mortality,[87] and the need for renal-replacement therapy in ICU settings.[87]

Calprotectin is an intracellular heterodimeric protein that, when secreted, acts as a damage-associated molecular pattern (DAMP) that can activate TLR4.[88] Its monomers are produced by collecting duct epithelial cells following postrenal AKI and by neutrophils following renal ischemia.[89,90] Neutrophil-secreted calprotectin promotes the recruitment of inflammatory monocytes and their differentiation into M1 macrophages.[90] However, following the initial injury, calprotectin plays a role in the prevention of renal fibrosis and promoting proper repair of the kidney. In patients undergoing renal surgery, calprotectin was increased at 2 h following injury and remained elevated for 5 days.[91] Calprotectin also accurately distinguished prerenal AKI from intrinsic AKI.[92] The clinical utility of calprotectin is limited by its upregulation by neutrophils in inflammatory states, such as urinary tract infections,[38] and by the kidney in urothelial carcinoma.[93]

Kidney injury marker-1 (KIM-1) is a transmembrane protein with an immunoglobulin-like domain that is expressed in a variety of cell types.[94] Following ischemia-reperfusion injury, KIM-1 is rapidly upregulated by proximal tubule cells and is shed into the urine in a matrix metalloproteinase-dependent manner.[94-99] The increased expression and shedding of KIM-1 makes it a sensitive and specific urinary biomarker for proximal tubule cell injury. KIM-1 binds to phosphatidylserine on apoptotic bodies and other cellular debris to mediate their phagocytosis.[100] In so doing, KIM-1 preserves renal function and attenuates the inflammatory response as demonstrated by mice expressing mutant KIM-1.[101] However, the severity of kidney injury impacts the efficiency of KIM-1 phagocytosis, as excessive shed KIM-1 can inhibit its own

debris clearance.[102] KIM-1 also binds to and antagonizes $G\alpha_{12}$ as a guanine dissociation inhibitor.[103] In AKI, $G\alpha_{12}$ disrupts tight junctions in a Src-dependent manner;[104] thus, KIM-1-mediated inhibition of $G\alpha_{12}$ preserved tubular structure in AKI.[103,105] However, in chronic kidney disease, KIM-1 expression correlated with a greater degree of inflammation and fibrosis.[106-108] Clinically, KIM-1, particularly in conjunction with IL-18, was an excellent biomarker of AKI following cardiac surgery and had predictive value for both intrinsic AKI and in-hospital mortality.[109] Because it is upregulated in a variety of proteinuric and inflammatory diseases,[107] its specificity for AKI can be limited in the clinic, but its sensitivity and specificity for tubular cell injury is ideal for experimental models testing interventions that modulate injury in various forms of AKI.[110,111]

Cell Types Involved in AKI

Most forms of intrinsic AKI are characterized by injury of the proximal tubule cell. The proximal tubule performs over 70% of solute reabsorption and secretion in the glomerular filtrate,[112] so the loss of its integrity has a highly detrimental effect on ion homeostasis and proper excretion of metabolic byproducts. Additionally, several key functional features of proximal tubule cells,[113] such as a brush border, drug metabolizing enzymes, and high mitochondrial content to accommodate their high energy demands, increase their susceptibility to nephrotoxic agents and other sources of injury.

Beyond the nephron, the kidney contains numerous other cell types, including endothelial, interstitial, and inflammatory cells (Figure 1-2). While the proximal tubule cell plays an important and often causative role in the initial injury in AKI, these other cell types play an important role in the propagation of injury and its resolution.

ENDOTHEIAL AND VASCULAR RESPONSES: Ischemic AKI causes increased production of endothelin-1, angiotensin II, thromboxane A2, prostaglandin A2, leukotrienes C4 and D4, and adenosine, as well as an increase in sympathetic nerve activation.[114-117] These responses lead to substantial vasoconstriction. There is also a decrease in production of vasodilatory molecules like acetylcholine, bradykinin, and nitric oxide.[118,119] Endothelial activation produces chemokines and adhesion molecules that recruit inflammatory cells such as neutrophils and monocytes.[120-122] These inflammatory cells release vasoactive cytokines and cause occlusion of small vessels by leukocyte-endothelial interactions and activation of the coagulation cascade.[120] Additionally, tubulo-glomerular feedback due to diminished sodium reabsorption in injured nephrons leads to further arteriolar vasoconstriction, further reducing glomerular filtration.[123] As the endothelium becomes damaged, its barrier function declines,[124] leading to interstitial edema. Following injury, an imbalance in angiogenic factors decreases the number of capillaries,[125,126] particularly on the inner stripe of the outer medulla. As a result, the

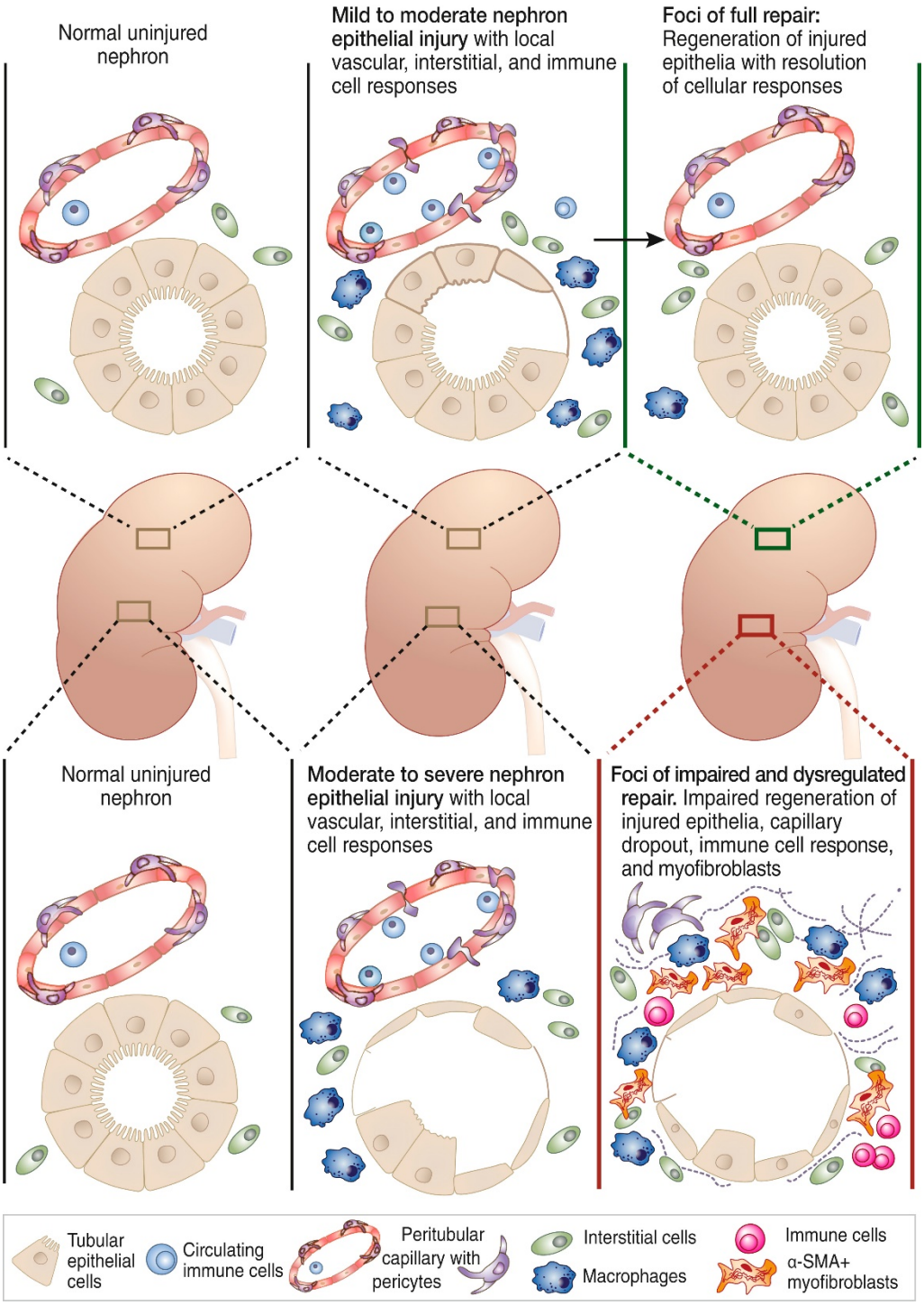


Figure 1-2. Involvement of numerous cell types in the pathogenesis of AKI. Following injury, endothelial and immune cells are activated and modulate injury progression and recovery from AKI. Different cell types are recruited depending on injury severity, and these cell types can negatively (neutrophils, M1 macrophages, fibroblasts) or positively (M2 macrophages, T_H2 cells, T_{Reg} cells) regulate recovery from AKI. Adapted from Kumar S. Cellular and Molecular Pathways of Renal Repair After Acute Kidney Injury. *Kidney Int.* 2017.

outer medulla is subjected to further hypoxia ,which sensitizes it to further injury and promotes maladaptive repair processes like fibrosis.[127]

INFLAMMATION: As renal cells are damaged in AKI, they release DAMPs that are recognized by the innate immune system to mount a systemic inflammatory response.[128] Following AKI, receptors for DAMPs, such as TLR2 and TLR4, were upregulated in renal cells.[129,130] Upon TLR activation, NFκB was activated to induce the expression of pro-inflammatory cytokines, such as TNF-α, IL-6, MCP-1, and IL-8.[131,132] These cytokines recruited other inflammatory cells, such as macrophages, neutrophils, and lymphocytes. HIF-1α activation within the kidney also enhanced macrophage infiltration following injury.[133] Additionally, activation of the endothelium (as described above) led to increased expression of adhesion molecules that recruit and activate immune cells. Once activated, immune cells secreted TNF-α and IFN-γ, which themselves can induce cell-death,[134] thereby leading to a necroinflammatory cascade.

Neutrophils, macrophages, dendritic cells, and lymphocytes are recruited to the kidney during the injury phase of AKI. Increased IL-8 secretion following injury increased neutrophil infiltration into the injured kidney.[135-137] Neutrophils also secrete IL-17, which promotes further neutrophil recruitment.[138] Once activated, neutrophils play a variety of roles,[139] including phagocytosis, ROS generation, cytotoxic peptide release, and NETosis, a process by which neutrophils release histones, DNA, and cytotoxic granules to trap and kill pathogens.[137] Although neutrophils have been observed in animal models of AKI and patient biopsy samples, targeting neutrophils to treat AKI has yielded mixed results.[140-142]

Macrophages, particularly of the M1 phenotype, contribute to early injury by recruiting neutrophils, secreting cytokines, and inducing apoptosis.[139] Systemic depletion of macrophages in mice attenuated renal ischemia reperfusion injury.[143,144] At later time points, M1 macrophages played a pro-fibrotic role, potentiating the AKI-CKD transition.[145,146] A switch to the anti-

inflammatory M2 phenotype protected against renal injury and promotes recovery following an insult.[147,148] This switch can be mediated by the increased proliferation of resident renal macrophages via CSF-1 or IRAK stimulation.[149-151]

While traditionally associated with delayed immune responses, various classes of lymphocytes play important roles in the development of AKI. Natural killer (NK) cells were activated by tubular osteopontin, leading to apoptosis of tubular epithelial cells.[152] NK T cells, which display both NK and T-cell markers,[153] can play a pathologic or a therapeutic role in AKI. NK T cells secrete pro-inflammatory cytokines such as IL-4, IL-10, and IFN-γ to recruit IFN-γ producing neutrophils.[154,155] General inhibition of NK T cells reduced the infiltration of IFN-γ producing neutrophils and attenuated renal injury.[156] However, specific inhibition or depletion of type II NK T cells increased injury, while activation of type II NK T cells by sulphatide attenuated injury.[157]

Regulatory T (T_{REG}) cells have been reported to have anti-inflammatory properties in numerous disease models. In the early phase of injury, T_{REG} cells secreted IL-10 to suppress the innate immune system and thereby attenuated renal injury.[158] T_{REG} cells trafficked to the kidney in the late injury phase,[159] whereupon they reduced inflammation and promoted tubular repair and regeneration.[158] Experimentally, inhibition or depletion of T_{REG} cells was detrimental to renal function,[158] while their expansion or activation promoted recovery.[160-162] However, the small number of T_{REG} cells in the kidney raises questions regarding the mechanism by which T_{REG} cells so potently modulate inflammatory responses despite their small population.[139]

$CD4^+$ and $CD8^+$ T cells are well-studied in multiple organ systems, and several therapies exist to selectively suppress these populations of inflammatory cells. T cell depletion by tacrolimus or inhibition of T cells with anti-CTLA-4 immunoglobulin reduced early injury in models of transplant-induced AKI.[163-165] Other work has shown that depletion of $CD4^+$ and $CD8^+$ cells protected against injury following ischemia reperfusion-induced AKI,[166,167] while adoptive

transfer of T cells restored injury. More specifically, CD4-knockout mice exhibited less injury following ischemia reperfusion,[167] and this protection was abrogated by adoptive transfer of $CD4^+$ T cells. Blockade of IL-16, a cytokine that recruits $CD4^+$ T cell recruitment, reduced not only T-cell infiltration but also renal injury.[168] Much like macrophages, $CD4^+$ T cells can exhibit multiple phenotypes. The inflammatory T_H1 phenotype is produced by STAT-4 activation and produces IFN-γ, while the anti-inflammatory T_H2 phenotype is produced by STAT6 activation to produce IL-4. STAT4-deficient mice (i.e., mice unable to generate the T_H1 phenotype) exhibited partial protection against AKI, but injury in STAT6-deficient mice (i.e., mice unable to generate the T_H2 phenotype) was potentiated.[169] While the role of $CD8^+$ cells in the pathogenesis of AKI has not been fully determined, evidence from germ-free mice showed that naïve $CD8^+$ T cells infiltrate the kidney following injury and produce IFN-γ to increase injury.[170] Even after the initial phase of injury, activated and memory T cells remained in the kidney,[171] suggesting that these cells may play a role in the pathogenic remodeling that leads to chronic kidney disease.

B lymphocytes (B cells) are recruited to the kidney following injury,[172] and mice deficient in B cells were protected from renal injury and exhibited more tubular repair during the recovery phase.[173] Adoptive transfer of B cells blocked the effects on recovery,[172] indicating that B cells may be a viable therapeutic target for AKI, particularly since most AKI patients are identified after the initial insult has occurred.

RENAL PROXIMAL TUBULE CELLS: Perhaps the central cell types in the pathogenesis of AKI is the renal proximal tubule cell (RPTC).[174] These cells are part of the renal epithelium and are responsible for the bulk of solute secretion and reabsorption into the urinary filtrate.[112] As a result, RPTC require a great deal of ATP and are therefore highly oxidative, containing numerous mitochondria.[175,176] However, this oxidative phenotype and high transport burden makes RPTC susceptible to injury following oxidative stress (such as by renal ischemia) or nephrotoxicant exposure. RPTC play important injury-sensing roles in the kidney and signal to

immune cells, endothelial cells, and fibroblasts during AKI to mediate injury and repair processes.[113] Importantly, transgenic mouse models that selectively injure or deplete RPTC have demonstrated that AKI severity correlates with the degree of RPTC loss.[177-179] Furthermore, RPTC injury and loss was sufficient to promote injury pathways in distal tubules and fibroblasts, underscoring the central importance of RPTC even in a multicellular pathology like AKI. These data were further corroborated by studies blocking p53-mediated apoptosis in RPTC,[180,181] wherein prevention of RPTC apoptosis protected against ischemic AKI.

Following AKI, RPTC underwent a loss of polarity with redistribution of transporters (e.g., Na^+/K^+ ATPase) and disruption of the actin cytoskeleton.[182] This was accompanied by a downregulation of mitochondrial metabolism and cell-cycle arrest to maintain cell membrane potential and energy balance. Additionally, integrins translocated from the basolateral to the apical surface (i.e., from basement membrane to tubular lumen).[183] As injury progressed, dead and intact RPTC sloughed into the tubular lumen and bound to these integrins, causing tubular obstruction and further injury. Damage- and pathogen-associated molecular patents (DAMPs and PAMPs, respectively) within the tubular debris were recognized by toll-like receptors, particularly TLR 2, TLR 4, and TLR 9.[184,185] These receptors were responsible for upregulation of inflammatory cytokines that enhance injury by reducing blood flow (leading to local hypoxia) and activating the innate immune system.[127] Additionally, RPTC lost expression of Crry,[186] a complement inhibitor, which led to increased inflammation and cell death. RPTC also regulated later immune responses by T-cells via MHC II expression.[187]

Following injury, RPTC upregulate the expression of injury markers, such as NGAL and KIM-1, described above. NGAL, along with other siderophores, scavenges iron to mitigate the propagation of oxidative stress.[188] KIM-1 acts a receptor for phosphatidylserine-mediated phagocytosis,[100] blocking pro-inflammatory signals and promoting clearance of luminal debris. However, chronic KIM-1 upregulation led to fibrosis by inducing MCP-1 and TGF-β expression

to recruit macrophages and fibroblasts.[106] This fibrotic signaling was exacerbated by Wnt,[189] Notch,[190,191] and TGF-β signaling pathways in the proximal tubules.[192,193]

Although proximal tubule cells are susceptible to injury, they also display substantial regenerative capacity. In the setting of mild ischemia, epithelial cells repolarized and returned to their healthy oxidative state.[194] Following more severe injury, loss of RPTC into the lumen necessitated regeneration.[195] RPTC dedifferentiate following injury,[196] while regenerated tubules have differentiated and functional RPTC. This finding raised questions of whether a stem or progenitor cell population might be responsible for tubular recovery. Examinations of lineage and clonal behavior have shown that terminally differentiated RPTC (rather than stem or extrarenal progenitor cells) were able to migrate, proliferate, and redifferentiate to generate functional tubules.[197,198] However, this repair was incomplete, as injured kidneys had shortened proximal tubule cells.[199] This finding may explain the increased susceptibility of patients with AKI to future bouts of AKI or development of CKD and ESRD.

Mitochondria and AKI:

The proximal tubules have an extensive role in the reabsorption and secretion of solutes from and into the glomerular filtrate. As a consequence, they consume a great deal of ATP and have a dense mitochondrial network. Disruption of or damage to this mitochondrial network led to morphological and functional deficits of the proximal tubules.[200] In addition to their role in generating ATP, mitochondria also play key roles in the regulation of ROS, steroid and heme biosynthesis, calcium and iron handling, and apoptosis.[176] The mitochondrial network is highly dynamic with mitochondria undergoing fission, fusion, and turnover (mitophagy) in response to cellular conditions. Therefore, mitochondrial dysfunction can lead to severe cellular consequences beyond the disruption of ATP-dependent processes.

Patients with mitochondrial diseases (that is, diseases due to mutations in the mtDNA or nuclear-encoded mitochondrial genes) often present with renal manifestations such as proteinuria,

metabolic acidosis, or progressive renal failure.[201,202] Histologically, their kidneys demonstrated tubular atrophy, interstitial nephritis, or glomerular involvement (such as focal segmental glomerular sclerosis).

Many causes of AKI, particularly intrinsic AKI, have associated mitochondrial dysfunction. Cisplatin inhibited the electron transport chain, diminished respiratory capacity and mitochondrial membrane potential, and activated mitochondrial cell death pathways.[203] Ischemia reperfusion injury led to an increase in ROS production,[204] which disrupted respiration and damaged the mitochondria, leading to subsequent cell death. Numerous models of AKI, such as sepsis,[205,206] glycerol-induced rhabdomyolysis,[207] folic acid,[208] and ischemia-reperfusion injury,[207] exhibited a persistent suppression of mitochondrial proteins, particularly subunits of the electron transport chain. Importantly, mitochondrial dysfunction preceded clinical manifestations of AKI,[209] suggesting that mitochondria play a causative role in the development of AKI. Samples from patients with various renal insults including trauma,[210] sepsis,[211] and ischemia have demonstrated swollen mitochondria and mitochondrial vacuolization, even with normal renal histology and function.[209]

Following AKI, proximal tubules exhibited increased mitophagy, or the selective degradation of mitochondria. Mitophagy occurs through two main pathways.[212,213] In one, the PINK1/Parkin pathway, PINK1 accumulates on the outer membrane of damaged mitochondria, where it binds the E3 ubiquitin ligase Parkin. Parkin then ubiquitinates mitochondrial proteins, which in turn bind to p62 and optineurin on the autophagosome. In the other pathway, the BNIP3/NIX/FUNDC1 pathway, BNIP3, NIX, and FUNDC1 on the outer mitochondrial membrane act as mitophagy receptors that bind LC3 on the autophagosome. By increasing mitophagic flux, cells can clear damaged and dysfunctional mitochondria to prevent excessive oxidative stress while maintaining ATP production. Both BNIP3 and PINK1 were increased following AKI,[214,215] and the induction of mitophagy was protective against AKI.[216,217] Mice fed

low-calorie diets had increased renal mitophagy with less oxidative stress,[218] leading to protection against AKI. Furthermore, activation of AMPK increased autophagy to protect against AKI by inhibiting mTOR, an inhibitor of autophagy.[219,220] However, direct inhibition of mTOR by rapamycin failed to prevent loss of renal function and inhibits tubular proliferation,[221-223] despite inducing autophagy. These effects were likely due to the role of mTOR in numerous cellular processes, including survival and proliferation.

Under normal conditions, mitochondria are dynamic organelles that undergo frequent fission and fusion events. Mitochondrial fission is mediated by dynamin-related protein 1 (Drp1) and its receptor, Fis1, while mitochondrial fusion is regulated by mitofusins 1 and 2 (Mfn1 and Mfn2, respectively) on the outer mitochondrial membrane and Opa1 on the inner mitochondrial membrane.[224] Following various organ injuries, including AKI, mitochondria became fragmented and swollen.[203] This was associated with an increase in Drp1 translocation to the mitochondria,[203,207] suggesting that modulating mitochondrial dynamics might be a therapeutic target for AKI. Indeed, inhibition of Drp1 by mDivi-1 prevented mitochondrial fission and apoptosis while also rescuing renal function following AKI.[203,225] Interestingly, mDivi-1 also had beneficial effects in mouse models of cardiorenal syndrome,[226] the major cardiovascular sequela following AKI. Based on the beneficial effects of inhibiting fission, the effects of increasing mitofusin expression on AKI have been studied. In vitro, deletion of Mfn2 enhanced RPTC susceptibility to apoptosis.[227] However, in vivo, proximal tubular deletion of Mfn2 increased proliferation through an ERK-dependent pathway and accelerates recovery of renal function.[228] In animal models of CKD, renal dysfunction and increased mitochondrial fusion were reversed by the antioxidant curcumin.[229] Together, these data indicate that while inhibition of mitochondrial fission is a promising therapeutic target, further study of mitochondrial fission and fusion is necessary to better implement therapies for AKI.

While mitophagy and mitochondrial fission decrease the mitochondrial pool, suppression of mitochondrial biogenesis (MB) also occurs after AKI. MB is the process by which new mitochondria are made and requires tight coordination between the nuclear and mitochondrial genomes. Following AKI, the expression of electron transport chain proteins was suppressed,[207,230,231] and ATP and mitochondrial function were decreased. One of the main regulators of MB is the transcriptional co-activator peroxisome proliferator-activated receptor gamma coactivator 1α (PGC-1α).[232] Following AKI, PGC-1α expression was decreased during the injury phase,[205,206] followed by an increase during the recovery phase. However, even in the recovery phase, the inactive acetylated form of PGC-1α was elevated.[233] Specific deletion of PGC-1α in RPTC worsened injury from AKI.[205] Overexpression of PGC-1α prevented TNFα-induced decreases in oxygen consumption and mRNA expression of mitochondrial genes.[205] However, in the setting of oxidant injury, overexpression of PGC-1α worsened injury but accelerated recovery of RPTC.[234] The latter data are of particular importance, as diagnosis of AKI often occurs after initial injury has occurred. Therefore, MB represents an attractive therapeutic target for AKI. Below, signaling pathways leading to MB and drugs that are known to induce MB will be discussed.

Inducing Mitochondrial Biogenesis to Treat Degenerative Diseases

Mitochondria, the metabolic powerhouses of the cell, have diverse functions including ATP production, biomolecule synthesis, ionic homeostasis and antioxidant defense. As cells age and accumulate damage, mitochondria less readily meet ATP demands, thereby diminishing the cells' functions and regenerative capacity. After toxicant exposure or cell stress, mitochondria can be damaged, and increased free radical production may be followed by persistent mitochondrial dysfunction. Diminished ATP and increased free radicals propagate injury and subsequent tissue and organ dysfunction (Figure 1-3). Indeed, many acute and chronic degenerative diseases across multiple organ systems are associated with a degree of mitochondrial dysfunction, often with suppression of electron transport chain proteins and activities.[235-238]

Because many diseases are associated with mitochondrial dysfunction, research is underway to develop therapeutics that target mitochondria to prevent disease progression. For example, numerous compounds have been studied that prevent cell death by interfering with the formation of the mitochondrial permeability transition pore (MPTP), reducing oxidative stress using mitochondrial-targeted antioxidants, or modulating mitochondrial dynamics by inhibiting mitochondrial fission or promoting mitochondrial networking.[239] However, whereas many of these strategies are effective for preventing injury in animal models, they target events that occur early in cellular dysfunction and therefore may be less efficacious for facilitating recovery after an insult. To address this problem, some groups have investigated compounds that induce MB, or the generation of new, functional mitochondria within cells to promote repair and regeneration.[235]

This section will describe the role of the peroxisomal proliferation activated receptor coactivator-1α (PGC-1α) in MB and the role of mitochondrial dysfunction in acute and chronic degenerative diseases. It will also describe existing compounds that induce MB, signaling pathways responsible for their effects, and finally, potential utility of these compounds for

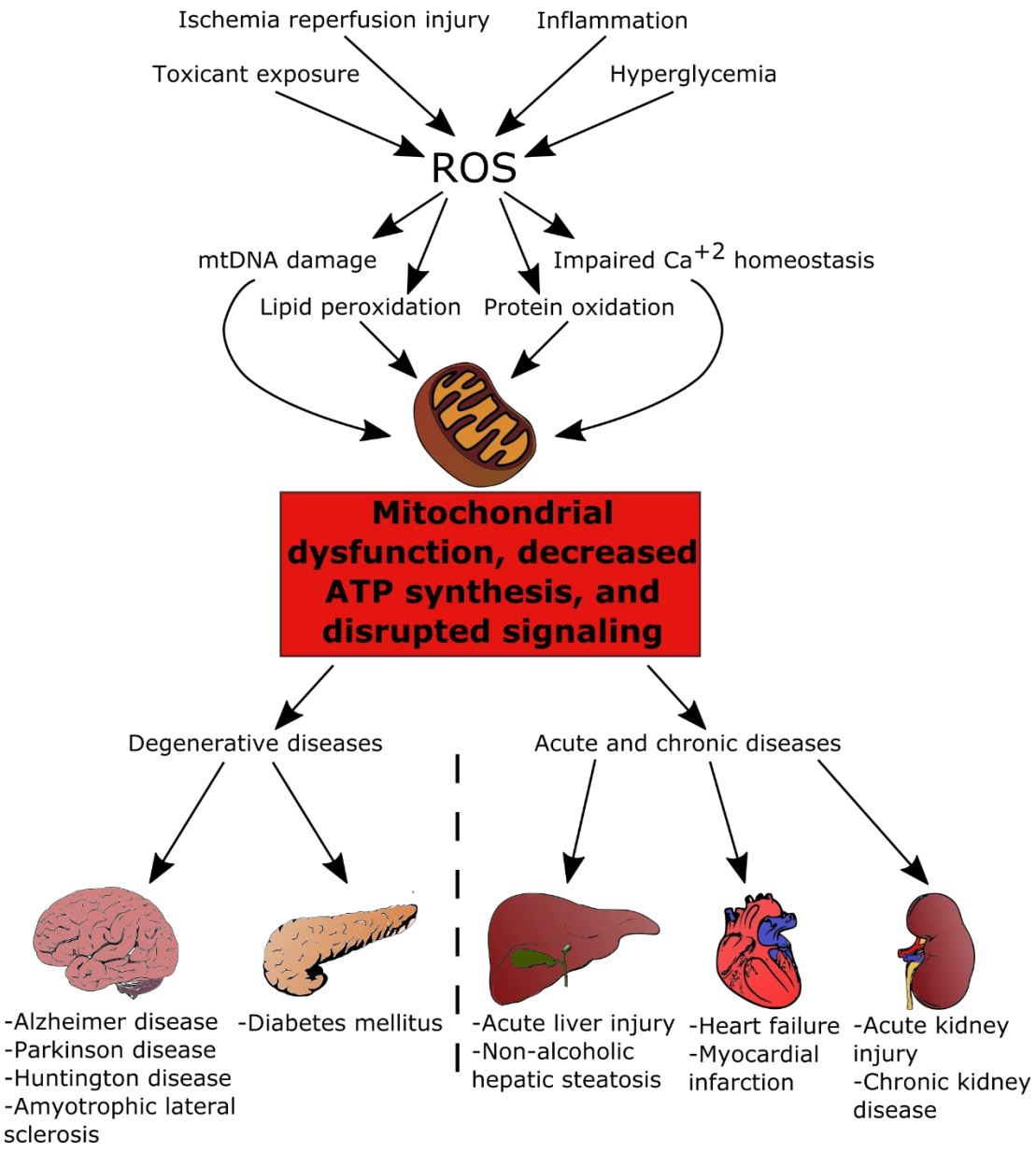


Figure 1-3. Multiple insults converge upon the mitochondria, leading to mitochondrial dysfunction and subsequent organ injury and disease. Following an insult, increased ROS generation leads to mitochondrial damage. Subsequent energy deficits and disrupted signaling lead to tissue and organ dysfunction.

treating human acute and chronic degenerative diseases for which there are presently limited therapeutic options.

Regulation of MB

MB requires the activation of a complex transcriptional and translational program integrating both nuclear and mitochondrial genomes.[240,241] Nuclear encoded mitochondrial genes, such as the mitochondrial transcription factors and the mitochondrial DNA (mtDNA) replication complex, facilitate transcription, replication, and proofreading of the mitochondrial genome.[240] Integrity of mtDNA replication is particularly important in aging and chronic degenerative diseases, where deleterious mtDNA mutations and deletions can lead to dysfunctional mitochondria.[242,243] For example, the nuclear transcription factors estrogen receptor (ER) and estrogen related receptor-α (ERRα), nuclear respiratory factors 1 and 2 (NRF-1 and NRF-2), peroxisome proliferator-activated receptor (PPAR) family of transcription factors, thyroid hormone receptor (TR), cAMP-responsive element binding protein (CREB), and yin yang-1 (YY-1)[244] increase expression of genes of the electron transport chain, mitochondrial transporters, antioxidant proteins, and other mitochondrial transcription factors. However, these transcription factors are pleotropic with effects on genes unrelated to MB. Selective induction of MB is typically regulated through transcriptional co-activation proteins such as the PGC-1 family (Figure 1-4). PGC-1 proteins activate transcription and translation of mitochondrial genes and increase energy production in healthy cells, whereas in injured cells PGC-1 activation often normalizes overall mitochondrial function as measured by ATP production, mitochondrial membrane potential, and reactive oxygen species (ROS) generation.[245-247]

The PGC-1 family, composed of PGC-1α, PGC-1β, and PGC-1 related coactivator (PRC), facilitate the formation of complexes capable of activating the transcription of nuclear genes related to MB.[248] PRC is thought to play a role in redox-sensitive inflammatory responses and MB during cellular proliferation, whereas PGC-1β appears to contribute more to maintenance

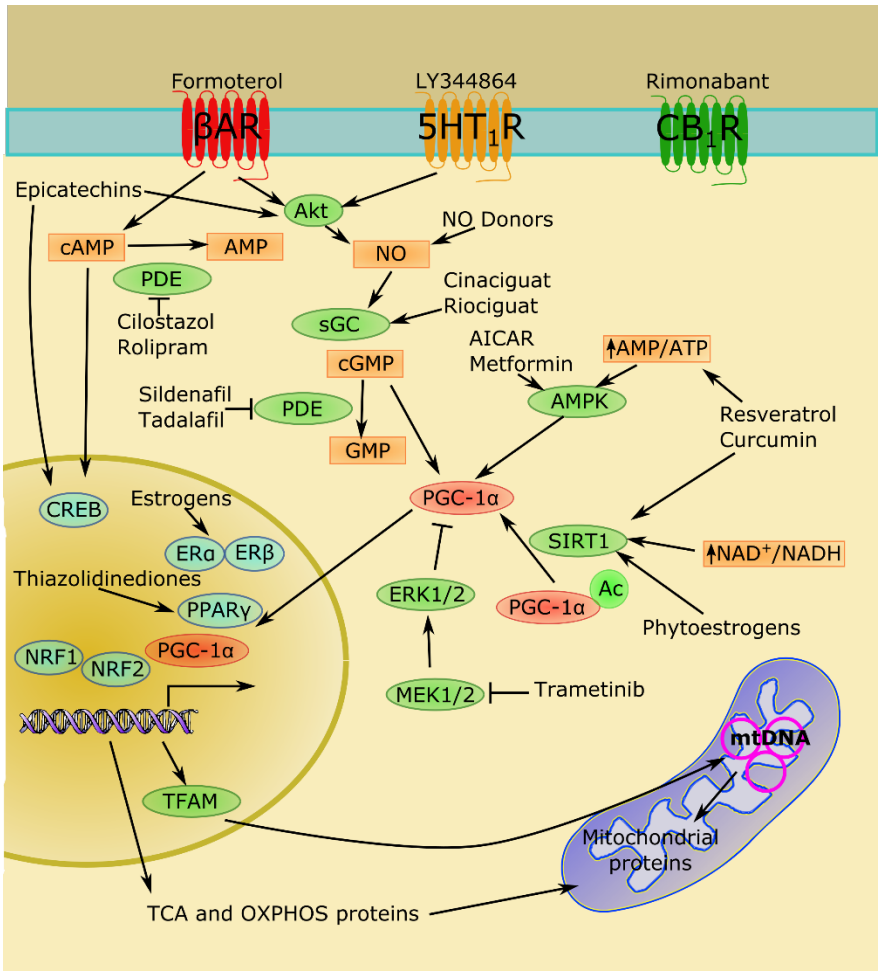


Figure 1-4. PGC-1α integrates extracellular and cytosolic signaling inputs to selectively upregulate mitochondrial biogenesis. Extracellular signals from GPCRs such as beta adrenergic receptors (βAR), serotonin receptors (e.g., $5HT_1R$), and cannabinoid receptors (e.g. CB_1R) modulate second messengers and kinase activity. These signals converge on transcription factors and PGC-1α to induce MB, with increased expression of tricarboxylic acid (TCA) and oxidative phosphorylation (OXPHOS) proteins. Various pharmacologic agents modulate these inputs and can therefore induce MB. Adapted from Cameron R.B. et. al. Development of Therapeutics That Induce Mitochondrial Biogenesis for the Treatment of Acute and Chronic Degenerative Diseases. *J. Med. Chem*. 2016.

of mitochondrial mass. In contrast, PGC-1α has been shown to drive MB in response to various environmental cues. Because PGC-1α tends to be the most inducible and responsive member of the PGC-1 family, its activation has emerged as a key therapeutic strategy for MB induction. However, it is important to note that PGC-1α-independent mechanisms of MB have been reported.[249-252] Such mechanisms include compensatory activation of PGC-1β or PRC and direct activation of transcription factors that induce mitochondrial genes.

Through activation of PGC-1α and its associated transcription factors, multiple signaling pathways have been shown to regulate MB. PGC-1α can be directly activated by silent mating type information regulation 2 homolog 1 (SIRT1)-mediated deacetylation,[253] methylation by protein arginine methyltransferase 1 (PRMT1),[254] or phosphorylation by kinases such as p38,[255] protein kinase A (PKA),[256] and AMP-dependent kinase (AMPK).[257] Additionally, PGC-1α and other transcription factors associated with MB can be activated by NO/cGMP and calcium-dependent signaling.[258] In summary, these diverse signaling inputs allow exquisite control of mitochondrial homeostasis to meet cellular energy demands and to maintain proper cellular function.

The Importance of MB in Disease

Because mitochondria regulate many processes within cells, mitochondrial dysfunction or disruptions in mitochondrial homeostasis lead to severe deficits in cellular functions.[235,236] Injury to mitochondria following ischemia reperfusion injury, toxicant exposure, or severe inflammatory response leads to deficient ATP and disruption of ion homeostasis. Additionally, mitochondrial stress increases superoxide anion production and which causes damage to proteins and lipid membranes. These mitochondrial derangements disrupt cellular repair, proliferation, and differentiation status and increase cell death.

Mitochondrial dysfunction has been implicated in numerous acute and degenerative disease processes, such as myocardial infarction,[259] stroke,[260] and AKI.[207] These disease states

may be attributed in part to the role of mitochondria and oxidative metabolism in cellular differentiation as observed in neurons,[261] myocytes,[262] and immune cells.[263] Chronic conditions causally linked to such acute insults (such as chronic kidney disease and heart failure) are also characterized by persistent mitochondrial dysfunction,[264,265] suggesting that the lack of mitochondrial recovery after an acute injury can also lead to chronic degenerative disease. For example, deficits in PGC-1 family proteins have been associated with the development heart failure in both animal models and human patients.[266,267] Interestingly, mice that overexpress PGC-1 proteins also exhibit abnormal cardiac function,[268] indicating that a tight control over mitochondrial content is necessary for normal organ function. Similarly, animal models of chronic kidney disease demonstrate diminished renal mitochondrial function,[269] and animal models of mitochondrial dysfunction demonstrate chronic kidney disease.[270] Finally, human patients with chronic kidney disease have decreased mtDNA in skeletal muscle and peripheral mononuclear blood cells,[269] suggesting that mitochondrial defects in a single organ can lead to global mitochondrial dysfunction.

Other chronic diseases also have been associated with disruption of mitochondrial homeostasis. Type II diabetes mellitus and metabolic syndrome are characterized by mitochondrial dysfunction associated with insulin resistance.[271] In metabolic syndrome, pancreatic beta cells exhibit increases in UCP2, decreased ATP synthesis, and increased levels of ROS.[272,273] Additionally, reductions in complex IV of the electron transport chain have been associated with the development of diabetes in obese mice and patients.[274] Furthermore, epigenetic silencing of electron transport chain genes and mtDNA,[275-277] along with genes associated with MB such as PGC-1α and TFAM,[278,279] lead to decreased mitochondrial content and a greater proportion of dysfunctional mitochondria, thereby causing sustained deficiencies in cellular respiration.

Multiple neurodegenerative diseases also have been associated with decreased mitochondrial mass, altered mitochondrial dynamics, and dysregulation of MB. Parkinson disease has been linked to a panoply of mutations that lead to mitochondrial dysfunction. Defects in PINK1 and Parkin disrupt clearance of damaged mitochondria, permitting accumulation of oxidative damage in dopaminergic neurons and suppression of PGC-1α and decreased cellular respiration.[280-283] Mutations in DJ-1 increase ROS while decreasing anti-oxidant defenses,[284] leading to decreases in mitochondrial membrane potential, poor mitochondrial quality control, and altered mitochondrial morphology. Similarly, mutations in mTDNA,[285-288] TFAM,[289] mortalin,[290] and α-synuclein[291] lead to increased susceptibility to ROS and subsequent mitochondrial dysfunction. Additionally, huntingtin mutants associated with Huntington's disease bind to the PGC-1α promoter and prevent its transcription and the transcription of other nuclear transcription factors associated with MB, including CREB.[292,293] Huntingtin mutations also cause impaired mitochondrial calcium handling,[294] reduced respiration,[295,296] and disrupted mitochondrial dynamics.[297,298] Finally, genetic and toxicant-induced models of Alzheimer disease and samples from human patients confirm the suppression of mitochondrial proteins and the MB transcriptome in Alzheimer disease,[299,300] along with mtDNA damage and disruptions in mitophagy and mitochondrial morphology.[301-303] Thus, compounds that induce MB may alleviate cellular dysfunction associated with acute and chronic degenerative diseases and promote organ repair and recovery that leads to improvements in patient health.[230]

Natural Products

Because mitochondria and oxidative stress are associated with aging, populations with longer lifespans have been studied to identify a potential means for preventing deleterious effects of aging. These studies have identified multiple chemicals capable of inducing MB (Figure 1-5), and these compounds have shown efficacy in multiple disease models by modulation of multiple

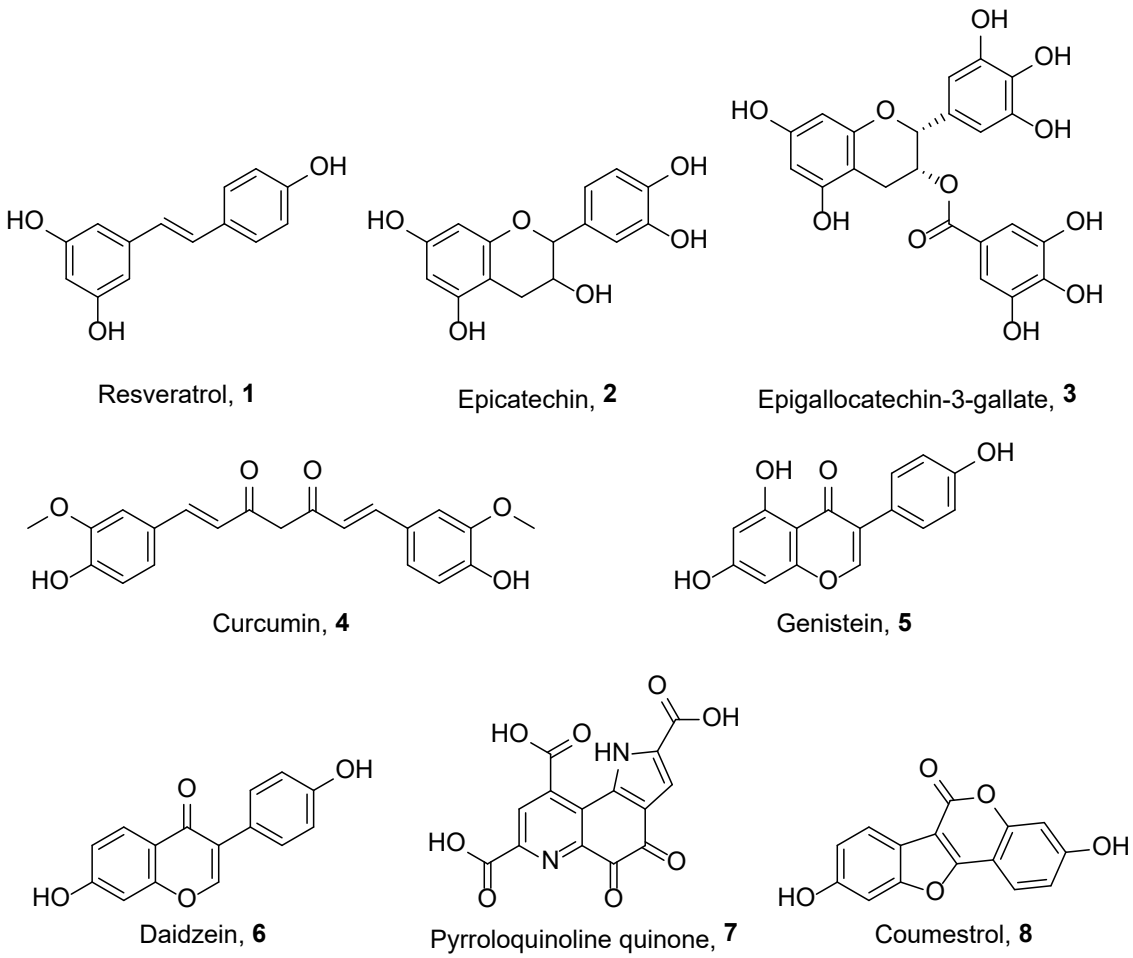


Figure 1-5. Naturally occurring polyphenols capable of inducing MB. Adapted from Cameron R.B. et. al. Development of Therapeutics That Induce Mitochondrial Biogenesis for the Treatment of Acute and Chronic Degenerative Diseases. *J. Med. Chem*. 2016.

signaling axes. Nonetheless, their therapeutic applicability in many cases is limited by poor absorption and low oral bioavailability.

Resveratrol

A widely studied nutritional activator of MB is the polyphenol resveratrol (**1**).[304] Compound **1** has been shown to induce MB by activating SIRT1 directly or indirectly through AMPK.[305] SIRT1 in turn deacetylates PGC-1α and allows it to exert its transcriptional effects. In particular, **1** activates AMPK by inhibiting components of the electron transport chain such as complex I and F1/F0 ATPase.[306,307] Docking studies with complex I suggest that resveratrol binds to the NAD^+ binding site of complex I through pi stacking interactions with its aromatic components and by hydrogen bond interactions through its hydroxyl group.[307] When binding F1/F0 ATPase, **1** prevents rotation of the ATP synthase complex through a network of hydrophilic and hydrophobic interactions.[306] Compound **1** can also directly activate PPARα via interactions with the 4'-hydroxyl group.[308] It also activates PPARγ by interactions between R280 and its 4'-hydroxyl group near the opening of the ligand binding pocket as well as Van der Waals interactions with F264, H266, and R288.[309] Together, protein-ligand interactions trigger signals that induce MB. In models of diabetic cardiovascular disease, **1** induces MB and restores vascular reactivity *in vitro* and *in vivo*.[310] In cellular and animal models of neuronal radiation damage,[311] Alzheimer disease,[312] Parkinson disease,[313] and Huntington's disease,[314] **1** normalizes mitochondrial function and rescue cellular viability and function. Compound **1** also attenuates oxidative stress in fibroblasts from patients with Complex I deficiency by increasing SOD2 in a SIRT3-dependent manner.[315] Human clinical trials using **1** demonstrated improved lipid profiles, antioxidant defenses, and vascular reactivity in diabetic and obese subjects;[316-321] however, there are conflicting data regarding the effect of **1** on insulin sensitivity,[316,320,322] and **1** had no effect in non-obese subjects.[323]

Epicatechins

(-)-Epicatechin (**2**),[324] primarily found in cocoa, has been shown to induce MB through multiple signaling pathways, including Akt-dependent nitric oxide (NO) generation,[325,326] CREB phosphorylation, and δ-opioid receptor activation.[327] The epicatechin epigallocatechin-3-gallate (**3**),[328] promotes cAMP-dependent signaling to increase SIRT1 and PGC-1α.[329] Although there are limited data regarding the structural basis for **2** activation of cAMP-dependent signaling, Akt-dependent signaling is mediated by the 3"-, 3'-, and 4'-hydroxyl groups.[330] Following oxygen-glucose deprivation, neuronal viability is rescued by **2** via the Akt-eNOS pathway and CREB activation.[326] In a mouse model of diabetes, **2** reduces oxidative stress in cardiac tissue by inducing MB.[331] Similarly, in mouse models of cardiovascular disease, **2** acts through the δ-opioid receptor to prevent mitochondrial swelling and to increase respiration;[327,332] it can also decrease cardiac ischemia-reperfusion injury through NO and cGMP generation. Even in aged mice, epicatechin increases expression of mitochondrial and antioxidant proteins.[333] Through its cAMP-dependent activation of SIRT1 and PGC-1α, **3** enhances MB in Down's syndrome patient fibroblasts and enhances mitochondrial calcium handling by modulating mitochondrial tethering to the rough endoplasmic reticulum.[329] Compound **2** also induces MB in human diabetic patients to improve skeletal muscle metabolism.[334]

Curcumin

Curcumin (**4**),[335] a diarylheptanoid found in turmeric, has shown promise for promoting MB and improved function in several disease models. By activating multiple signaling molecules, including p38, PKA, AMPK, SIRT1, and NRF2, **4** can induce MB and protect cells against injury.[336-338] The *o*-methoxy group in compound **4** is important for increasing p38-mediated HO-1 expression, which confers cytoprotection in endothelial cells.[336] The unsubstituted 5'- and 5''-positions and its olefinic system allow **4** to inhibit NF-κB and activate the NRF2 pathway.[339] In cellular models of metabolic syndrome, **4** rescues hepatic mtDNA, NRF1, and TFAM and

reduces inflammation and NFκB activity.[340] In white adipose tissue, **4** increases browning and markers of MB via increases in norepinephrine and β_3 adrenergic receptor expression.[341] Pretreatment with **4** improves mitochondrial membrane potential, oxygen consumption rates, and survival in cellular models of Parkinson disease.[342] Compound **4** attenuates neuronal death and reduces infarct size following cerebral ischemia-reperfusion injury with concomitant increases in mitochondria and improvements in neurological function.[343] In animal models of metabolic syndrome, **4** restores hepatocyte mitochondrial function to reduce hepatosteatosis.[344] Following gentamicin-induced nephrotoxicity, **4** can increase PGC-1α and NRF2, thereby elevating mitochondrial protein expression and improving mitochondrial structure.[337] In rat skeletal muscle, **4** increases mtDNA content and mitochondrial protein expression following endurance training via PKA-dependent activation of AMPK, SIRT1, and PGC-1α.[338]

Phytoestrogens

Phytoestrogens, such as genistein (**5**),[345] daidzein (**6**),[346] pyrroloquinoline quinone (**7**),[347] coumestrol (**8**),[348] and equol (**9**),[349] are natural products often found in legumes such as soybeans. They have been shown to exert their effects in part by modulation of estrogen receptors and partly via activation of SIRT1.[350-352] 5-hydroxyl groups prevent SIRT1 activation, whereas 7-hydroxyl groups are necessary for SIRT1 activation. Similarly, a 3-phenyl group appears to drive increased SIRT1 expression.[352] Compounds **5**-**8** have been shown to induce MB *in vitro*.[352-354] Additionally, through their biogenic effects, **5** and **6** rescued cultured RPTC from oxidant injury.[352] *In vivo*, **5** and **9** induce MB to improve bioenergetics in ovariectomized mice.[355,356] Both **5** and **6** increase mitochondrial markers with associated improvements in insulin sensitivity and glucose metabolism in diabetic mice.[357,358] Compound **5** also reduces the size of a myocardial infarct in mice by rescuing mitochondrial function.[350] Finally, **7** stimulates MB in both wild type mice and transgenic models of Alzheimer disease;[359,360] in the latter model, improvements in synaptosomal bioenergetics are correlated with cognitive improvement.

Transcription Factor Modulators

Although natural products have been useful in identifying biological targets for MB, their poor pharmacokinetic parameters limit their therapeutic potential. Modulators of the transcriptional machinery responsible for MB can potently and efficaciously induce MB; however, because they activate transcriptional programs other than MB, these compounds can have severe side effects that limit their clinical utility.

Thiazolidinediones

The thiazolidinediones (TZDs) are a class of hypoglycemic drugs used to treat diabetes mellitus that includes rosiglitazone (**10**),[361] pioglitazone (**11**),[362] troglitazone (**12**),[363] and ciglitazone (**13**) (Figure 1-6).[364] Classically, they act as agonists of the transcription factor peroxisome PPARγ, leading to increased insulin sensitivity. These effects are primarily mediated by the acidic head group, which engages in necessary hydrogen bonding interactions with PPARγ to stabilize its active conformation.[365,366] More recently, acute PPARγ-independent effects of TZDs have been discovered, including inhibition of the electron transport chain, which reduces the ATP/AMP ratio, leading to AMPK activation and subsequent MB.[367-369] TZDs have also been shown to exert anti-inflammatory effects and to upregulate the mitochondrial stress-response, leading to increased anti-oxidant defenses.[367] Although they upregulate multiple signaling pathways, the capacity of TZDs to sensitize tissues to the effects of insulin has been shown to correlate with increased expression of mitochondrial proteins, suggesting that induction of MB may be central to the clinical efficacy of these drugs.[370] *In vitro*, **10**-**13** increase cell viability and improve neuronal function in models of ischemic injury,[371] Alzheimer disease,[372] Huntington's disease,[373,374] and multiple sclerosis.[375] Similarly, in animal models of neurodegenerative diseases, **10** and **11** improve both cellular and behavioral markers of neurological function.[376,377] In animal models of cardiac disease, **10** can rescue cardiac increases cardiac ROS and can be arrhythmogenic.[378,379] In models of metabolic syndrome, **10**-**13** induce MB in adipose tissue,[249,380]

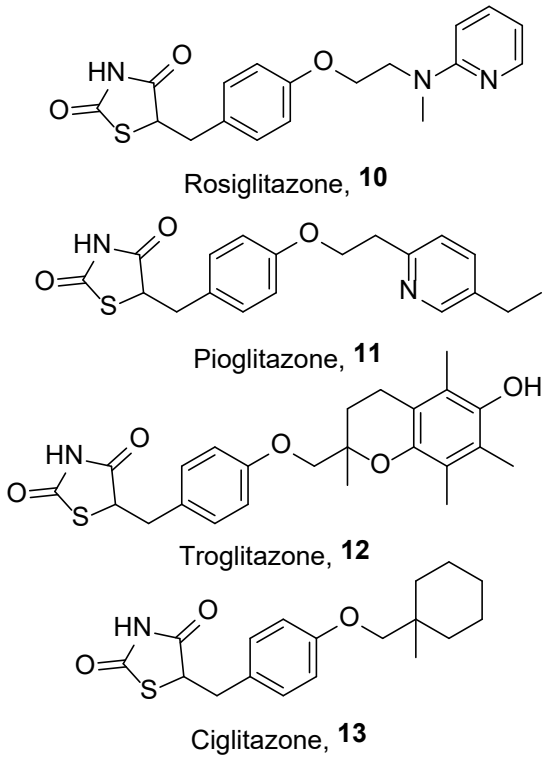


Figure 1-6. Thiazolidinedione inducers of MB. Adapted from Cameron R.B. et. al. Development of Therapeutics That Induce Mitochondrial Biogenesis for the Treatment of Acute and Chronic Degenerative Diseases. *J. Med. Chem*. 2016.

pancreatic beta cells,[381] and skeletal muscle[369,382] to enhance insulin sensitivity. In humans, **11** induces MB in subcutaneous adipose tissue,[383] and **10** can do so in skeletal muscle.[384]

Estrogens

To understand the underlying processes responsible for sex-dependent differences in lifespan and oxidative stress, multiple groups reported that estrogens can be protective in various tissues. Furthermore, reduced levels of estrogens, such as in ovariectomized mice, lead to increased ROS production.[385] Estrogens (Figure 1-7) can bind to the transcription factors estrogen receptor α (ERα) and estrogen receptor β (ERβ) to directly influence gene expression. 17β-Estradiol (**14**)[386] and progesterone (**15**)[387] are the principle biologically active estrogens. **14** and **15** interact with nuclear estrogen receptors by hydrogen bonding interactions between the ligands' hydroxyl groups and the receptors' polar residues and by hydrophobic interactions with the receptors' binding pockets.[388] ERα-selectivity, such as by the selective ligand 4,4',4''-(4-propyl-[1*H*]-pyrazole-1,3,5-triyl)*tris*phenol (**16**),[389] is mediated by steric bulk to interact with a residue found in ERα but not ERβ.[389] Selectivity for ERβ by diarylpropionitrile (**17**)[390] is mediated by phenolic groups, while its efficacy is improved by its nitrile group.[390] Recently, it has also been shown that estrogens activate plasma membrane-bound estrogen receptors such as the G protein-coupled estrogen receptor (GPER). The GPER-selective ligand G-1(**18**)[391] is structurally similar to **14** but is unable to form hydrogen bonds in the nuclear estrogen receptors;[391] however, **18**'s acetyl group and pseudosymmetry allows engagement of specific residues of the GPER to stabilize the active conformation.[392,393] Compound **14** has been shown to induce MB in immortalized cell lines and in a cellular model of Leber hereditary optic neuropathy, a mitochondrial disease. [394,395] In animal models, **14** normalizes ROS production, increases antioxidant defenses, and enhances respiratory capacity in the heart and brain.[385,396] Furthermore, **15** and synthetic estrogen receptor agonists such as **16** and **17** have been shown to enhance respiratory capacity in the brain and promote clearance of lipid ERα and ERβ differentially regulate the expression of electron transport chain

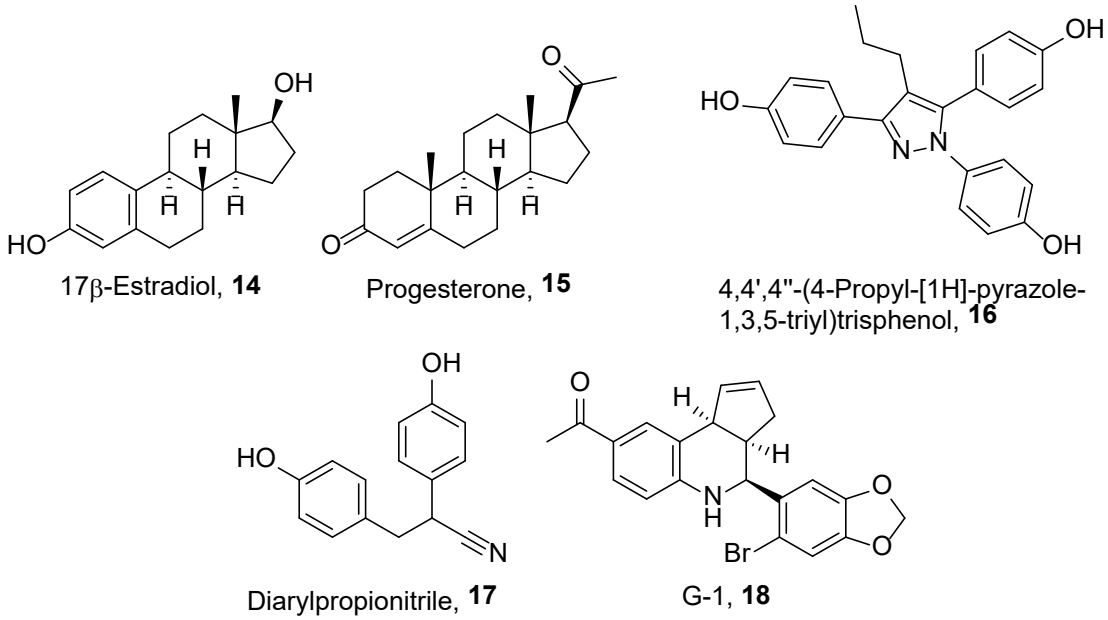


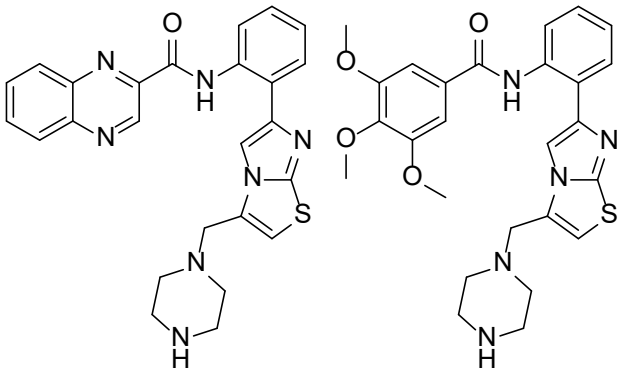
Figure 1-7. Estrogen inducers of MB. Adapted from Cameron R.B. et. al. Development of Therapeutics That Induce Mitochondrial Biogenesis for the Treatment of Acute and Chronic Degenerative Diseases. *J. Med. Chem*. 2016.

proteins. Additionally, at least a portion of the cardioprotective effects of estrogen are mediated through the GPER, as shown by stimulation with the GPER-selective agonist **18**.[385] Despite the clear protective potential of estrogens, their proliferative and endocrine effects limit their use as a long-term therapy for chronic degenerative diseases. However, the development of selective ER and GPER ligands that drive specific signaling and transcriptional programs may improve the utility of such therapeutics.

SIRT1 activators

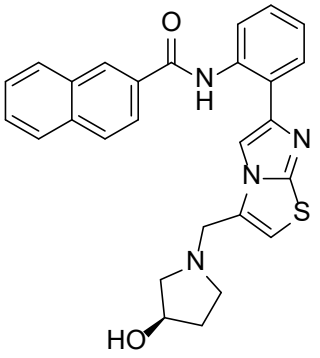
The identification of SIRT1 as a common target of natural product-induced increases in PGC-1α led to the development of multiple SIRT1 activators, such as SRT1720 (**19**),[397] SRT1460 (**20**),[397] SRT2183 (**21**),[397] and SRT2104 (**22**) (Figure 1-8).[398] In the initial synthesis of SIRT1 activators,[399] the basic methylamino ring at C-3 of the imidazothiazole ring of **19** and **20** enhanced water solubility, while derivatization of the amide group (such as with the 2-quinoxaline group of **19**) improved potency and efficacy. Interestingly, both **19** and **20** share a methylamino ring and have greater efficacy, whereas **19** and **21** have a 2-quinoxaline group and more potency,[397] suggesting that the two groups may play distinct roles in the pharmacodynamic qualities of these compounds. The direct mechanisms of action for the sirtuin class have been controversial. Assays with isolated fluorescent peptides were used for optimization, but direct proteomic assays indicate that **19-21** do not directly activate SIRT1 and, rather, act promiscuously to activate or inhibit numerous targets;[400] however, other work has shown that these compounds directly activate SIRT1 by binding to amino acid E230.[401]

Due to numerous SIRT1 targets, these activators can affect various cellular processes, including inflammation, lysosomal trafficking, and metabolism. Among its targets, SIRT1 deacetylates PGC-1α, facilitating nuclear import of and transcriptional regulation by PGC-1α, leading to MB. In models of type II diabetes mellitus, SIRT1 activators have been shown to improve lifespan, normalize pancreatic morphology, improve insulin, glucose, and fatty acid

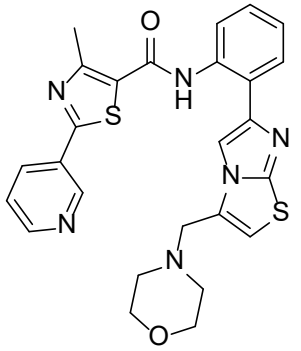


SRT1720, **19**

SRT1460, **20**



SRT2183, **21**



SRT2104, **22**

Figure 1-8. Activators of SIRT1 that induce MB. Adapted from Cameron R.B. et. al. Development of Therapeutics That Induce Mitochondrial Biogenesis for the Treatment of Acute and Chronic Degenerative Diseases. *J. Med. Chem*. 2016

metabolism and increase mitochondrial markers;[397,402-404] however, other studies have shown a lack of efficacy in diabetic mice, calling into question the beneficial effects of these compounds.[400] With respect to neurodegenerative diseases, SIRT1 activators prevent neurodegeneration and restore MB in animal models of Huntington's disease and multiple sclerosis.[405,406] SIRT1 activation has shown promise in renal disease, restoring renal function after AKI and preventing renal medullary damage in obstructive nephropathy.[233,407,408] In models of cardiovascular disease, **19** reduces the size of myocardial infarction and preserves contractility,[409] as well as reducing ROS and improving contractility in mice with enhanced ALDH2 activity.[410] Compound **19** also preserves endothelial function in aged mice.[411] Even in healthy animals, **19** and other SIRT1 activators have been shown to extend lifespan and "healthspan" by preventing the development of age-associated diseases in multiple organ systems.[412] In human trials, **22** improved lipid profiles in diabetic patients but did not affect plasma glucose or insulin, likely due to large pharmacokinetic variability.[413] Additionally, **22** reduces cholesterol, LDL, and triglycerides in otherwise healthy smokers,[414] suggesting that SIRT1 activation is important to the human healthspan.

Kinase Modulators

Kinases either phosphorylate target proteins or function as scaffolds to co-localize other kinases and targets to regulate cellular signaling. Phosphorylation of specific targets can either activate or inhibit cellular signaling pathways in response to environmental cues. Because they are central signaling molecules, kinases are attractive therapeutic targets. In particular, activators of kinases that induce MB, such as AMPK, can be useful in multiple diseases. Unfortunately, inhibitors are easier to develop, and most kinase modulators are inhibitors. However, inhibitors of kinases that negatively regulate MB, such as extracellular signal-regulated kinases 1/2 (ERK1/2), also provide promise as therapeutics.

AMPK

AMPK is an energy sensing kinase involved in the modulation of metabolism through the cellular AMP/ATP ratio. AMPK activation is increased during exercise and induces MB, and it is decreased with aging and during multiple chronic degenerative diseases.[415] AMPK activation has been shown to be an upstream regulator of sirtuins and therefore PGC-1α.[416] Furthermore, pharmacologic activation of AMPK has been observed with multiple natural products that induce MB. Activators of AMPK (Figure 1-9), including the indirect activators AICAR (**23**),[417] metformin (**24**),[418] phenformin (**25**),[419] R419 (**26**),[420] and C24 (**27**),[421] and the direct activator A769662 (**28**),[422] have been developed and induce MB in multiple cell lines. Additionally, **23** has been shown to enhance proliferation and increase ATP in models of complex I deficiency and MELAS.[423,424] Compound **23** is biotransformed via phosphorylation within the cell and acts as an AMP mimetic to activate AMPK and other AMP-dependent processes.[417] The biguanides **24** and **25** activate AMPK in a LKB1-dependent manner and through inhibition of complex I;[420,425] by inhibiting the electron transport chain, the AMP/ATP ratio is increased, leading to AMPK activation. Compound **26** also indirectly activates AMPK via complex I inhibition,[420] and **28** activates AMPK by binding to an allosteric site between the alpha and beta subunits of AMPK. **28** both allosterically activates and prevents Thr172 dephosphorylation.[426]

In models of diabetes and metabolic syndrome, **23** mimics high intensity exercise in skeletal muscle with accompanying increases in SIRT1 activation and PGC-1α activity. These improvements in MB decrease oxidative stress in both renal and endothelial cells,[427-429] preventing common comorbidities such as diabetic nephropathy and poor wound healing. Compound **23** can also improve pancreatic morphology via AMPK activation to enhance insulin sensitivity and GLUT4 expression,[430] thereby decreasing plasma glucose. In hepatic cells, **27** reduces lipid biosynthesis to prevent lipid accumulation and preserve hepatic

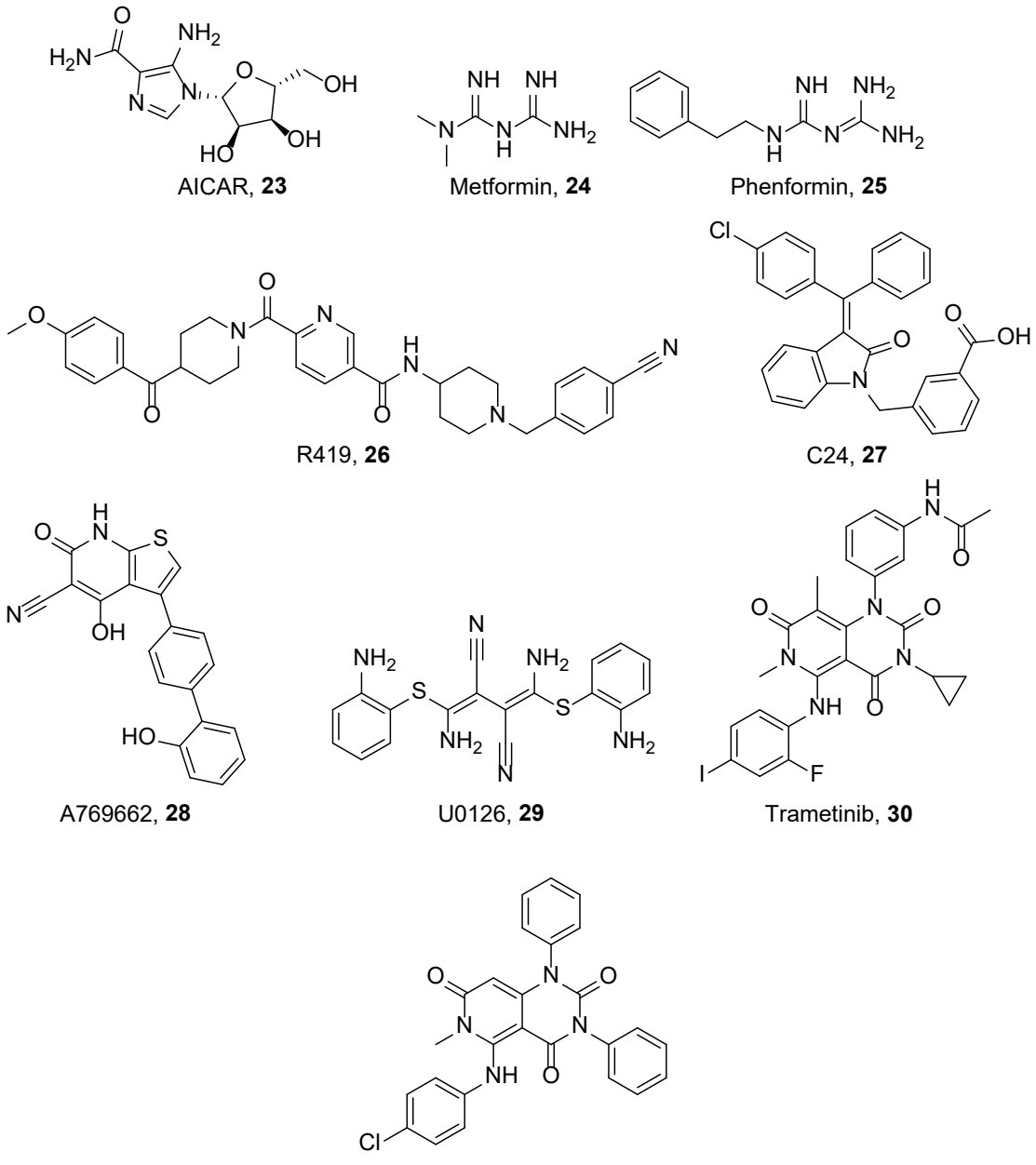


Figure 1-9. Kinase modulators that induce MB. Adapted from Cameron R.B. et. al. Development of Therapeutics That Induce Mitochondrial Biogenesis for the Treatment of Acute and Chronic Degenerative Diseases. *J. Med. Chem*. 2016.

function.[421] In humans with gestational or type II diabetes, **23** and **25** prevents insulin resistance in multiple tissues.[431-433] In the heart, **23** reduces oxidative stress and improves contractility,[434] and it is associated with improvements in insulin sensitivity in diabetic mice as well as reductions in cold ischemic injury in mouse models of heart transplant.[435]

AMPK activators have also shown promise for treating neurodegenerative diseases. Neuronal activity has been shown to drive PGC-1α and NRF-1 expression in an AMPK-dependent manner,[436] leading to MB, and pharmacologic activation of AMPK has been shown to mimic these effects. Compound **23** has also been shown to impact neuronal development by promoting mitochondrial accumulation at axonal branch points, thereby facilitating branch formation and retention.[437] In models of Alzheimer disease, **23** ameliorated mitochondrial dysfunction and prevented neurotoxicity and tau hyperphosphorylation.[438,439] Compound **23** decreased amyloid beta, a protein implicated in Alzheimer disease, in a PPARγ dependent manner.[440] Compound **23** has also been shown to decrease inflammation in models of multiple sclerosis, attenuating pathological and behavioral changes. Furthermore, in models of ischemic brain injury, **23** diminishes ischemic neuronal damage.[441]

ERK1/2

Another means of inducing MB is the inhibition of negative regulators of MB, such as ERK1/2. Following its activation by MEK1/2, ERK1/2 regulates a variety of cellular processes, including differentiation, apoptosis, survival, proliferation, and motility.[442] Inhibition of MEK by U0126 (**29**)[443] or trametinib (**30**)[444] leads to a rapid suppression of ERK1/2 phosphorylation (Figure 1-9). Compound **29** can exist in the (*Z,Z*) or (*Z,E*) isomer; however, the (*Z,Z*) isomer provides better MEK inhibition, as does the presence of electron donating amino groups at *o*-positions of its phenyl groups.[443] The iodo- and cyclopropyl groups of Compound **30** improve potency for cancer cell growth inhibitory activity over its lead compound JTP-70902 **(31)**[444], while its methyl groups improve stability and its acetamide group improves solubility.[444] ERK1/2

has been shown to suppress PGC-1α in melanoma cells.[445] Additionally, in models of Parkinson disease ERK1/2 activation leads to phosphorylation of TFAM, impairing its ability to bind to mitochondrial DNA.[446] MEK1/2 inhibitors, such as **29** and **30**, have been developed for cancer chemotherapy. *In vitro* models of renal oxidative stress indicate that ERK1/2 is a mediator of oxidative damage in proximal tubule cells, and that its inhibition by **29** prevents oxidative damage.[447] Our laboratory has shown that ERK1/2 activation increases after AKI and that pre-treatment with the MEK1/2 inhibitor **30** rescues mitochondrial function and restores renal function in a mouse model of AKI.[206] These data indicate that inhibition of suppressors of MB can induce MB and restore organ function following injury.

Cyclic Nucleotide Modulators

The cyclic nucleotides cGMP and cAMP are cellular second messengers that are generated in response to extracellular signals. They activate downstream kinases or are hydrolyzed by phosphodiesterases (PDE). NO increases cGMP synthesis by binding to a heme group on soluble guanylate cyclase (sGC), while cAMP is increased through activation of adenylate cyclase by the stimulatory G-protein $G\alpha_s$. Because cyclic nucleotide generation is disrupted in multiple pathological states, cyclic nucleotide modulators are attractive targeted therapies for the induction of MB in various diseases.

NO-cGMP-PKG Axis

The NO-cGMP-PKG pathway can be modulated by: 1) nitric oxide (NO) donors, such as sodium nitroprusside (**32**), (±)S-nitroso-N-acetylpenicillamine (SNAP, **33**),[448] diethylamine NONOate (DEA-NONOate, **34**),[449] and diethylenetriamine-NONOate (DETA-NONOate, **35**)[449] which increase cellular NO (Figure 8); 2) sGC stimulators and activators, such as cinaciguat (**36**),[450] riociguat (**37**),[451] and BAY 41-2272 (**38**)[452] which directly increase cGMP production (Figure 1-10); and 3) phosphodiesterase (PDE) inhibitors, such as zaprinast (**39**),[453] sildenafil (**40**),[454] udenafil (**41**),[455] tadalafil (**42**),[456] and vardenafil (**43**)[457] which increase cGMP by

preventing its hydrolysis (Figure 1-10). Clinically, these compounds are used to induce vasodilation to treat hypertension or erectile dysfunction. Activation of this pathway has been shown to increase PGC-1α and stimulate MB both through the activation of PKG and nitrosylation of transcription factors to increase their binding to the PGC-1α promoter.[458,459]

As their name implies, all NO donors have a group, usually a nitrate or a furoxan group, that can be liberated to form NO. Because the NO donating group is small, NO donors can be "fine-tuned" for multiple clinical uses and to slow the rate of NO release.[460,461] However, because NO generation causes such a dramatic drop in blood pressure, NO donors are of limited clinical use. However, these compounds readily confirm the importance of NO for preventing metabolic derangements and cell death, particularly in skeletal muscle. In hypoxia, dietary nitrate (a natural NO donor) prevents PGC-1α suppression, leading to increases in fatty acid oxidation and respiration. Even under normoxic conditions, nitrate stimulates MB in a cGMP/PKG-dependent manner.[462] Compound **33** has also been shown to induce MB in myoblasts and reduce the effects of caspase-dependent and –independent apoptotic molecules,[463] and **34** also improves synaptic conduction in models of Alzheimer disease in a cGMP-dependent manner.[464] sGC activators and stimulators increase the activity of sGC in the absence of NO. Stimulators such as **37** and **38** increase sGC activity with a non-oxidized heme group, whereas activators increase sGC activity even if the heme prosthetic group is oxidized. Both classes of compounds have been approved for clinical use to treat pulmonary hypertension. Compound **38** was optimized for vasorelaxation through the addition of a 2-fluoro-phenyl group, a pyrazolo[3,4-*b*]pyridine ring, and a cyclopropyl group.[452] Compound **37** was optimized to increase oral bioavailability and half-life, and to reduce clearance via amino and N-methylcarbamate substitutions on the pyrimidine group.[451] On the other hand, sGC activators have shown greater utility beyond blood pressure control, likely due to their capacity to activate sGC even under high oxidative stress. Compound **36** was identified using a high-throughput screen and was confirmed to displace the heme of sGC

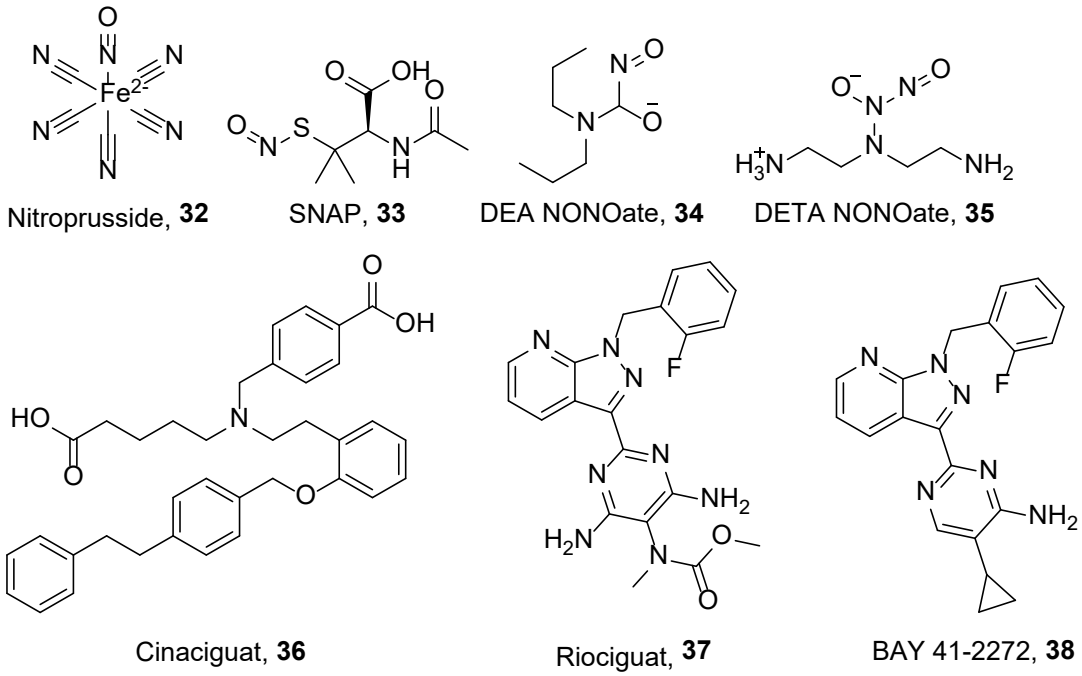


Figure 1-10. Activators and stimulators of the NO/cGMP pathway. Adapted from Cameron R.B. et. al. Development of Therapeutics That Induce Mitochondrial Biogenesis for the Treatment of Acute and Chronic Degenerative Diseases. *J. Med. Chem*. 2016.

by interacting with its YXSXR motif through carboxylic acid moieties.[465] In pre-clinical studies, compounds **36**-**38** improve cardiac, renal, and neurological function across multiple disease models including ischemia reperfusion injury, sepsis, diabetes, and Alzheimer disease.[466-470] However, despite the efficacy of cGMP in promoting MB, few studies have examined the role of MB in these functional improvements. Compound **36** protects against myocardial infarction by increasing H_2S, a known inducer of MB,[471] suggesting that further investigation is warranted into the role of MB in these compounds' protective effects.

Inhibition of cGMP-selective PDEs prevents cGMP hydrolysis, promoting its accumulation in the cell and facilitating stimulation of MB. Compound **40** was designed from **39** by mimicking the guanosine dipole moment, adding an ethoxy group to improve potency, and adding a piperazine sulfonamide to improve solubility, selectivity, and potency.[472] However, both **40** and **41** discriminate poorly between PDE5 and PDE6, leading to visual side effects.[455] Compound **42** has better selectivity for PDE5 over PDE6 with the addition of more electron donating groups; however, relative to **40** and **43**, **42** is less selective for PDE11.[456,473,474] Although these compounds have been extensively developed for treating pulmonary hypertension and erectile dysfunction, they also have been tested for treating other diseases as described below.

Because cGMP-selective PDE inhibitors were designed to reduce blood pressure via increased vasodilation, it is reasonable that they have been tested for conditions characterized by endothelial dysfunction, such as diabetes. As expected, in models of diabetes, **40** improves endothelial function as measured by flow-mediated dilation.[475,476] In addition to their effects on vascular reactivity, **40**, **42**, and **43** reduce plasma markers of diabetes, such as lipids, serum glucose, and HbA_{1c}, and are associated with improvements in mitochondrial content.[477-480] In adipocytes and hepatocytes, **40** enhances lipid oxidation and increases insulin tolerance and cellular morphology.[477] cGMP-selective PDE inhibitors also reduce diabetic complications in other organs, such as the kidney and heart. In models of diabetic nephropathy, **40** reduces

microalbuminuria, a predictor of renal and cardiac dysfunction.[478] Additionally, in diabetic mice, **42** rescues the expression of cardiac cytoskeletal and redox proteins to improve cardiac morphology and function.[480,481]

In addition to beneficial reductions in the development of diabetic cardiomyopathy, cGMP-selective PDE inhibitors also ameliorate non-diabetic cardiac dysfunction. In ischemic cardiomyopathy and myocardial infarction, **40**, **42**, and **43** increase survival and decrease infarct size by reducing cell death and preserving mitochondrial function.[482-484] Compound **42** also prevents cardiac remodeling and hypertrophy, stabilizing contractility rather than allowing progression to heart failure and pulmonary edema.[485] Similarly, in models of mitral regurgitation and doxorubicin toxicity, **40** inhibits cell death and preserves mitochondrial function by upregulating anti-apoptotic proteins and maintaining the mitochondrial membrane potential.[486,487]

cAMP-PKA-CREB axis

CREB regulates PGC-1α activity and expression to promote MB and is down-regulated in multiple disease states characterized by mitochondrial dysfunction. In Alzheimer disease, CREB phosphorylation is diminished due to impaired activation by PKA. This loss of activity leads to a downregulation of PGC-1α and an imbalance in tau protein, a driver of Alzheimer disease.[488] A similar decrease in CREB activity has been observed in Huntington's disease.[489] Additionally, ethanol decreases cellular cAMP, thereby reducing CREB activity to suppress PGC-1α and thereby exert its toxic effects.[490] Taken together, these data indicate that activation of the cAMP-PKA-CREB signaling pathway can promote MB and protect against neurodegenerative diseases.

The primary therapeutic approach for activating this signaling axis is with phosphodiesterase (PDE) inhibitors such as rolipram (**44**)[491] and cilostazol (**45**)[492] (Figure 1-11). Compound **44** inhibits PDE4, a cAMP-selective PDE, whereas **45** inhibits PDE3, a PDE capable of hydrolyzing both cAMP and cGMP; however, PDE3's V_{max} for cAMP is substantially higher

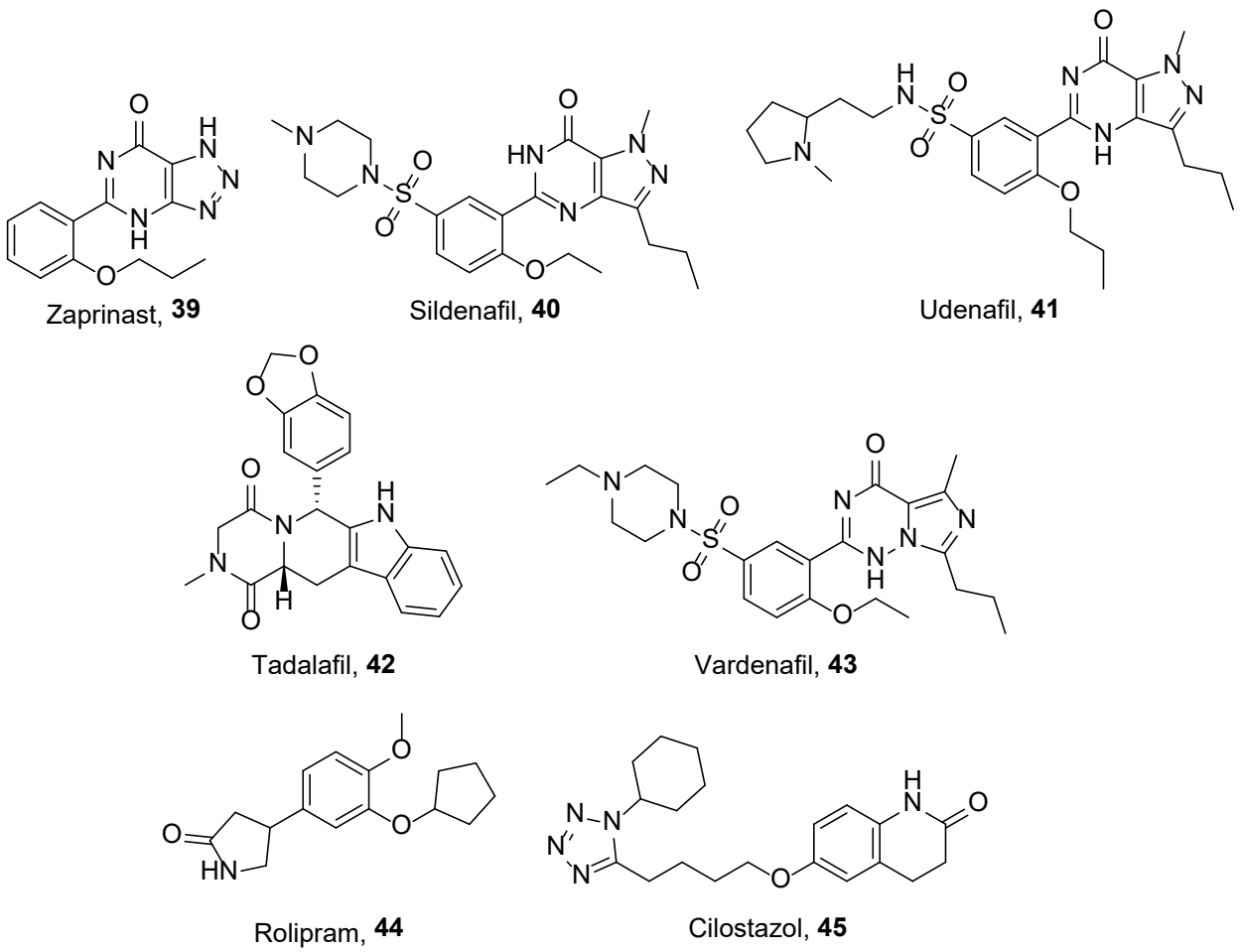


Figure 1-11. Phosphodiesterase (PDE) inhibitors associated with MB. Adapted from Cameron R.B. et. al. Development of Therapeutics That Induce Mitochondrial Biogenesis for the Treatment of Acute and Chronic Degenerative Diseases. *J. Med. Chem*. 2016.

than that of cGMP. Compound **44**'s selectivity arises in part from its optimized potency for PDE4 and the unfavorable orientation of a conserved glutamate residue in other PDEs.[493] In contrast, the lactam group of **45** engages in hydrogen bonding interactions with multiple receptor residues to promote PDE3 selectivity.[494] Both **44** and **45** can increase PGC-1α *in vitro*, indicating that they induce MB,[495] and both have shown potential for therapeutic use in pre-clinical disease models. However, in humans, **44**'s narrow therapeutic window limits its application, whereas **45** is approved for clinical use in the treatment of diabetic vascular complications.

Restoration of the cAMP-PKA-CREB pathway substantially reduces the effects of neurodegenerative diseases. In animal models of Huntington's disease, **44** improves neuronal function, morphology, and survival and decreases neurological impairment.[489,496] Compound **44** also reduces synaptic conduction abnormalities associated with Alzheimer disease, improving cognition.[497,498] These effects and increased CREB phosphorylation lasted beyond the cessation of treatment. In ischemic brain injury, **45** reduces neuroinflammation, reducing infarction size and decreasing apoptosis and free radical production.[499,500] In models of Alzheimer disease, **45** increases SIRT1 expression, reducing symptoms and improving cognitition.[501] Furthermore, in a retrospective study, **45** improved cognition in human patients,[502] suggesting that PDE3 inhibition holds promise for treating Alzheimer disease. Used clinically to treat claudication, the beneficial effects of **45** in models of diabetic cardiovascular disease are well studied. In models of limb ischemia, **45** increases angiogenesis by rescuing PPARγ, increasing angiogenic factors vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF);[503,504] this normalization of PPARγ also occurs in other tissues, such as the retina and the kidney.[505] Compound **45** also prevents endothelial cell senescence by increasing cAMP, leading to SIRT1 activation. In the heart, **45** reduces oxidant-induced mitochondrial dysfunction and significantly reduces myocardial infarction size.[506-508] Furthermore, **45** improves insulin sensitivity and reduces blood

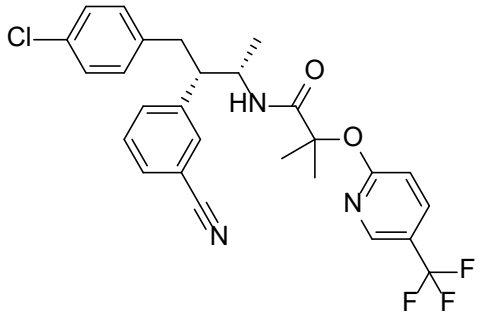
glucose and HbA_{1c} in diabetic mice and human subjects,[509-511] as well as reducing the urinary excretion of albumin and renal inflammation, indicating that **45** improves diabetic nephropathy. Despite these promising data, controversy exists regarding use of cAMP-selective PDEs in chronic degenerative diseases of the liver and kidney. On the one hand, **45** improves hepatic function after ischemic insult by inducing MB;[512] however, in models of lipotoxicity, increased cAMP acts synergistically to induce cell death despite concurrent stimulation of MB.[513] Additionally, despite the promising work in diabetic nephropathy described previously, we found that cAMP-selective PDE inhibitors do not induce MB in proximal tubule cells,[514] suggesting they are poor therapeutic options for treating AKI.

GPCR Ligands

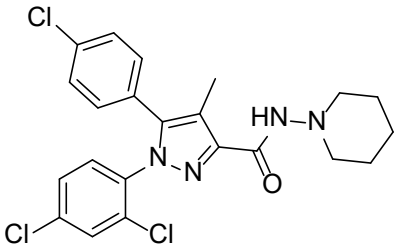
G protein-coupled receptors (GPCRs) are well characterized plasma membrane receptors that are the target of a substantial portion of currently available drugs. By coupling to G proteins, GPCRs can modulate cAMP, calcium, and NO and activate various kinases and signaling pathways. Additionally, different ligands of the same receptor can cause activation of distinct signaling programs, a phenomenon known as "functional selectivity" or "biased agonism."[515] By stabilizing different receptor conformations, different ligands can alter receptor interactions with G proteins, G protein-coupled receptor kinases (GRKs), and scaffolding proteins such as arrestins. One such scaffolding protein, GRK interacting protein 1 (GIT1), regulates MB in the heart, likely in an eNOS-dependent manner.[516,517] Biased agonism allows for the development of ligands that selectively stimulate signaling pathways that lead to MB while inhibiting negative regulators of MB. Many GPCRs are modulated by endogenous molecules, a fact which has facilitated the development of potent and selective agonists and antagonists for various receptors. Despite the potential of GPCRs to activate pathways known to induce MB and the availability of clinically approved GPCR ligands, little investigation has occurred to explore the potential of such compounds to induce MB.

Cannabinoid-1 receptor

Cannabinoid-1 receptor (CB1R) antagonists such as taranabant (**46**)[518] and rimonobant (**47**)[519] were studied for anorectic effects (Figure 1-12). Despite the lack of a cyclic linker, **46** binds in a similar mode to **47**; however, the amide group on **46** is able to engage in an extra hydrogen bonding interaction, leading to its enhanced affinity for the CB1R.[518,520] By inhibiting CB1R activity in the brain, these compounds can suppress appetite and cause weight loss with concomitant improvements in plasma lipid profiles. Both **46** and **47** were efficacious for inducing weight loss in wild type mice, mice fed a high fat diet, and ob/ob mice.[521,522] Inhibition of CB1R by **47** or by genetic ablation induces MB in adipose tissue and MB in a cAMP- and eNOS-dependent manner, leading to decreases in body weight and fat content.[521] Interestingly, **47** increased mitochondrial energy production was not correlated with increased mitochondrial mass in rat livers, indicating improved mitochondrial efficiency.[523] Although both **46** and **47** were efficacious in animal models, investigation of **46** was halted in Phase III trials, and **46** was withdrawn from the market in the U.S. after initial approval as an anti-obesity drug. In humans, **47** reduced food intake and increased energy consumption to promote weight loss but caused serious side effects such as suicidal ideation and severe depression.[524] [525]



Taranabant, **46**



Rimonabant, **47**

Figure 1-12. Cannabinoid-1 Receptor antagonists. Adapted from Cameron R.B. et. al. Development of Therapeutics That Induce Mitochondrial Biogenesis for the Treatment of Acute and Chronic Degenerative Diseases. *J. Med. Chem*. 2016.

5-Hydroxytryptamine receptors

Endogenous serotonin binds to the 5-hydroxytryptamine (5-HT) class of receptors (**48**, Figure 1-13)[526]. 5-HT receptors are primarily GPCRs that have been identified as therapeutic targets for neuropsychiatric, neurologic, and cardiac diseases. The synthetic ligand alpha-methyl-5-hydroxytryptamine (**49**)[527] possesses an extra methyl group that prevents its metabolism by monoamine oxidase.[528] The 5-HT_2 receptor agonist DOI (**50**)[529] has enhanced selectivity due to its primary amine, with the iodo-group adding to its potency.[530] Much work has been done to identify and characterize the pharmacophore of 5-HT_{2C} receptor agonists (e.g., CP809101, **51**)[531] and antagonists (e.g. SB242084, **52**)[532] and optimize their selectivity.[530-532] 5-HT_{2C} receptor agonists stabilize the TM6 domain of the receptor through its aromatic group, whereas antagonists interact with Asn331, Val354, and Ser334 through a positively ionizable group.[533]
In addition to direct 5-HT receptor antagonists, serotonin reuptake inhibitors such as fluoxetine (**53**)[534] prevent the uptake and degradation of **48** and prolong its actions at its receptors. The *p*-trifluoromethyl group of **53** confers selectivity for the serotonin reuptake transporter by binding to I172 in its transmembrane domain.[535,536] Treating rat pups with **53** improves mitochondrial membrane potential, respiratory capacity, and antioxidant defense in the heart, implicating **48** in mitochondrial health during development.[537]

Our laboratory identified multiple ligands that induce MB through various 5-HT receptors. In RPTC, we have shown that the non-selective 5-HT receptor agonist **49** induces MB.[538] The 5-HT_2 receptor agonist **50** increased cellular respiration *in vitro* and improved recovery from oxidant injury by *tert*-butyl hydrogen peroxide (TBHP); interestingly, induction of MB did not reduce initial injury by TBHP.[539] The 5-HT_{2C} selective ligands **51** and **52** induce MB *in vitro* and in naïve mice; interestingly, siRNA studies and work in knockout mice indicate that the ligands exert these effects through the 5-HT_{2A} receptor.[540]

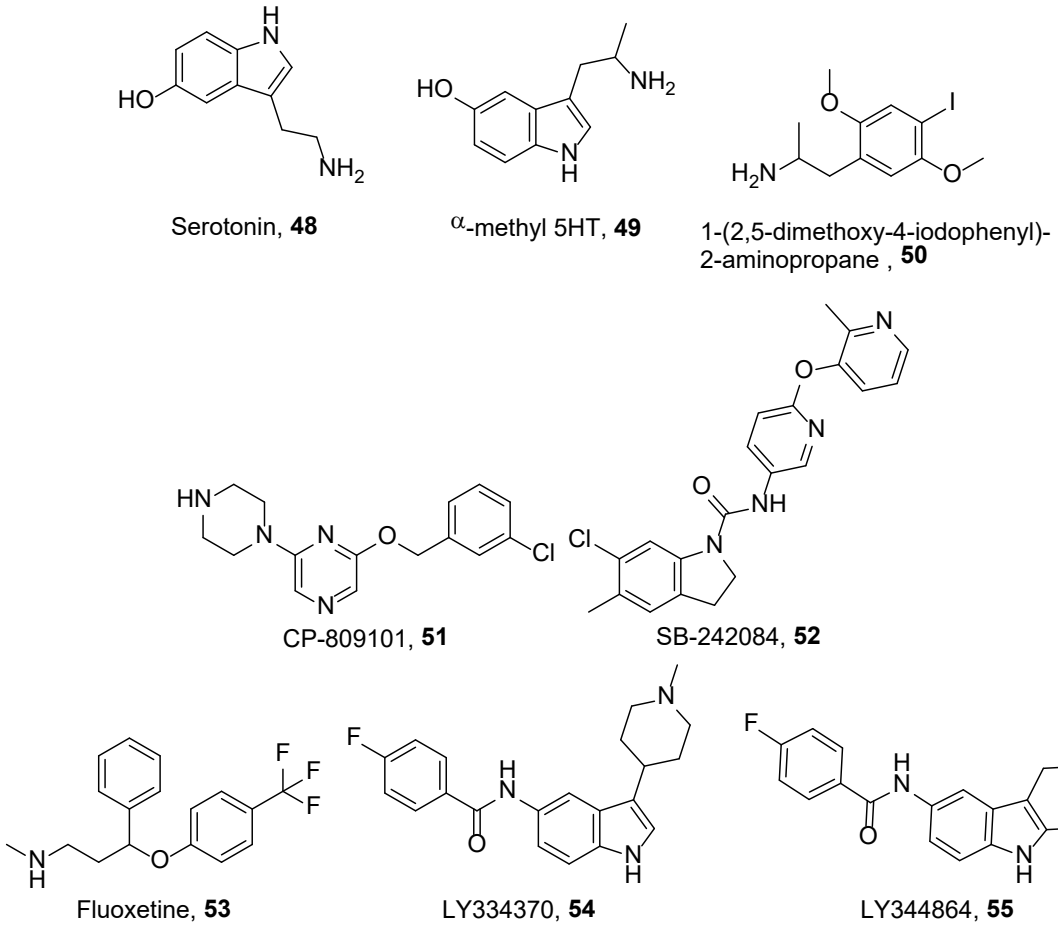


Figure 1-13. 5-Hydroxytryptamine receptor modulators that induce MB. Adapted from Cameron R.B. et. al. Development of Therapeutics That Induce Mitochondrial Biogenesis for the Treatment of Acute and Chronic Degenerative Diseases. *J. Med. Chem*. 2016.

In contrast to 5-HT_2 receptors, the 5-HT_{1F} receptor has few selective ligands-namely, LY334370 (**54**) and LY344864 (**55**) and limited data regarding its pharmacophore. Nevertheless, the selective 5-HT_{1F} agonists **54** and **55** induced MB *in vitro*, and **55** also improved recovery from ischemia-reperfusion-induced AKI *in vivo*.[538] Additionally, preliminary data suggest that **55** stimulates MB through the Gβγ-dependent activation of Akt and eNOS.[541] These data indicate that the induction of MB by 5-HT agonists could be clinically useful for treating AKI and other acute organ injuries as they effectively promote recovery and regeneration even after initial injury.

Beta adrenergic receptors

The beta adrenergic receptor family is activated by endogenous stress hormones epinephrine (**56**)[542] and norepinephrine (**57**, Figure 1-14)[542] and the family comprises three receptors. First, the beta-1 adrenergic receptor, primarily expressed in the heart, is targeted by drugs that affect cardiac contractility and heart rate. The beta-2 adrenergic receptor (β_2AR), which is ubiquitously expressed, is a target of bronchodilators to treat asthma and COPD. The beta-3 adrenergic receptor, which is primarily expressed in adipose tissue and the urinary bladder and is targeted to treat overactive bladder.[543]

Beta-adrenergic agonists contain distinct structural features, specifically a catechol or phenethanolamine core, whereas antagonists have a 3-aminophenoxypropan-2-ol core. However, while beta-adrenergic agonists have been extensively studied to optimize pharmacodynamics and pharmacokinetic parameters, there are few studies relating structural features to the induction of MB. Compounds **56**, **57**, and the non-selective beta adrenergic receptor agonist isoproterenol (**58**)[544] increase PGC-1α in brown adipose of naïve mice and in models of obesity in a cAMP- and p62-dependent manner.[545] Interestingly, in models of cardiac dysfunction, beta-1 adrenergic receptor stimulation by dobutamine (**59**)[546] increases cell death and inflammation,[547] but its

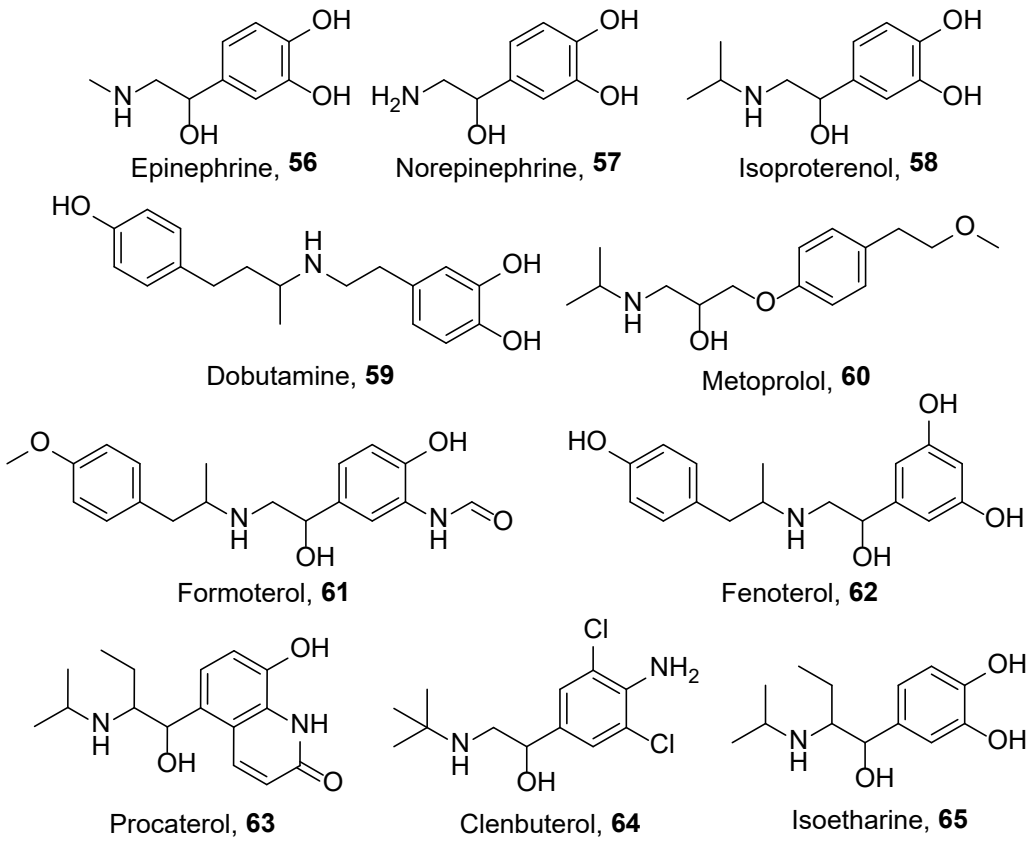


Figure 1-14. Beta adrenergic receptor modulators tested for the induction of MB. Adapted from Cameron R.B. et. al. Development of Therapeutics That Induce Mitochondrial Biogenesis for the Treatment of Acute and Chronic Degenerative Diseases. *J. Med. Chem*. 2016.

blockade by the beta-1 selective antagonist metoprolol (**60**)[548] enhances PGC-1α activation and improves cardiac metabolism and function.[549,550]

Beta-2 Adrenergic Receptors and Mitochondrial Biogenesis

Our laboratory has studied β_2AR selective agonists in renal MB, and the following section will discuss the signaling of the β_2AR and its known role in the induction of MB.

The β_2AR is a well-studied and well-drugged GPCR. The β_2AR is ubiquitously expressed and is activated by its endogenous ligand, epinephrine, to induce smooth muscle relaxation in the vasculature and airways,[551,552] increased glycogenolysis in the liver,[553] inhibition of histamine-release by mast cells,[554] and increased insulin secretion from the pancreas.[555] More relevant to the kidney, the β_2AR regulates systemic blood pressure and solute reabsorption by increasing renin secretion,[556] thereby activating the renin-angiotensin-aldosterone system.

The β_2AR canonically couples to $G\alpha_s$, leading to an increased accumulation of cAMP and subsequent activation of PKA, Epac, and CREB.[557] However, the β_2AR has also been shown to couple to $G\alpha_i$, which inhibits adenylate cyclase and releases the $G\beta\gamma$ heterodimer, and arrestins, which act as scaffolding for other effector molecules. Phosphorylation of the C-terminal tail of the receptor by G protein-coupled receptor kinases (GRKs) and other kinases such as PKA altered coupling of these effectors. The phosphorylation of the receptor by these kinases can lead to a switch from $G\alpha_s$ to $G\alpha_i$,[558] thereby limiting the duration and magnitude of cAMP-dependent signaling, or it can lead to the recruitment of β-arrestin,[559] which modulates desensitization, receptor internalization, or its own signaling program.

The coupling of these effectors depends on the conformation of the receptor, which, in turn, is dependent on the structure of the bound ligand. Thus, different ligands binding to the same receptor, either at allosteric or orthosteric sites, can cause differences in downstream signaling.[560] This phenomenon is known as “functional selectivity” or “biased agonism.” β_2AR agonists have been shown to be G protein biased (that is, selectively activating G protein-depending signaling cascades) and arrestin biased (that is, selectively activating an arrestin

signaling program).[557] In addition to effects on effector coupling to the receptor, biased ligands can also impact the signaling properties of the effector molecules. For example, different ligands of the calcitonin receptor altered the conformation of the receptor-G protein complex,[561] leading to differences in cAMP accumulation. A similar phenomenon has been observed for arrestins,[562] with different ligands leading to distinct conformational signatures that inform function.

A phenotypic screening for compounds that induce MB identified formoterol as a potent and efficacious inducer of MB in RPTC. Because the β_2AR is so widely drugged, other β_2AR agonists were screened for their ability to induce MB in RPTC. In particular, formoterol (**61**),[563] fenoterol (**62**),[564] and procaterol (**63**)[565] induced MB *in vitro* at pharmacologically relevant doses.[566,567]. However, other β_2AR agonists such as clenbuterol (**64**)[568] and isoetharine (**65**)[569]did not induce MB *in vitro*,[566] suggesting that ligand-directed signaling can be exploited to develop more effective mitochondrial biogenic β_2AR agonists.

Beta adrenergic signaling activates PGC-1α and induces MB in multiple tissues. In Leydig cells, stress increased steroid hormone production,[570] a process involving mitochondrial enzymes. This was associated with increased mitochondrial content and the expression of PGC-1 proteins through cAMP/PKA/CREB, NO/cGMP, and beta adrenergic receptor signaling. In mouse skeletal muscle and in human subjects, exercise induced MB in a beta adrenergic receptor-dependent manner.[571,572] While isoproterenol did not affect MB in human skeletal muscle,[573] in mice the effects on PGC-1α and MB were mimicked by treatment with clenbuterol.[571] Both exercise and clenbuterol specifically increased the b and c isoforms of PGC-1α in skeletal muscle,[571] while other environmental inducers (fasting and cold) caused increases in tissue-specific expression patterns of PGC-1α isoforms.[574] The tissue-specific changes in PGC-1α isoforms suggest differences in signaling for MB across cell types that would explain differences in β_2AR agonist efficacy for MB. Consistent with this hypothesis, in rats clenbuterol activated

p38 MAP kinase to induce MB in fat but not skeletal muscle, despite activation of CREB in both tissues.[575]

Formoterol (**61**) has been confirmed to induce MB *in vivo* in naïve mice as well as in mice subjected to AKI,[231] and this was associated with improvements in renal function, indicating that formoterol has therapeutic promise for treating AKI. Because both MB-inducing and non-MB-inducing β_2AR agonists increased cAMP, we suggest that the classical $G\alpha_s$-signaling pathway is not responsible for β_2AR-induced MB in the kidney. In addition to its renal effects, formoterol inducds MB in multiple other tissues, including the heart and skeletal muscle.[567,576] Together, these data indicate that certain β_2AR agonists such as formoterol can be used to treat multiple diseases and improve mitochondrial function and ameliorate symptoms.

Previous studies have explored β_2AR activation as a therapeutic target for AKI. In RPTC cell lines primarily expressing the β_2AR, isoproterenol stimulated proliferation in a cAMP-dependent manner,[577] which would be beneficial following RPTC loss in AKI. Following injury by shigatoxin, the β_2AR agonist terbutaline inhibited caspases to prevent apoptosis.[578] Furthermore, overexpression of the β_2AR reduced injury and promotes recovery from septic AKI,[579-581] while blockade of the β_2AR worsened septic AKI.[582,583] Despite these findings, excessive β_2AR activation reduced creatinine clearance in rats.[584] Together, these studies indicate that selective and limited activation of the β_2AR is a viable therapeutic strategy for AKI. However, the aforementioned studies focused on regulation of proliferation, inflammation and apoptosis, particularly in settings of β_2AR overexpression.

Our laboratory sought to determine the role of β_2AR activation in a more physiologically relevant model of AKI. Mice subjected to bilateral renal ischemia/reperfusion injury developed AKI with peak injury at 24 h and a persistent loss of renal function at 144 h.[207,231] These mice also exhibited persistent suppression of MB and oxidative phosphorylation. Administration of

formoterol after development of AKI improved renal function and rescued mitochondrial protein expression at 144 h.[231] However, because the β_2AR is ubiquitously expressed, and because various cell types mediate the pathogenesis of AKI,[194] it was unclear through which cell type(s) formoterol exerts its effects. Furthermore, as the β_2AR activates numerous signaling pathways, and as not all β_2AR agonists induced MB, the mechanism by which formoterol induced MB remained unclear.

Our previous studies showed that formoterol induced MB in primary cultures of RPTC,[567] while the selective β_2AR agonist clenbuterol did not.[566] Therefore, we chose clenbuterol as a pharmacologic probe for β_2AR signaling that does not induce MB in RPTC. Furthermore, in vivo, RPTC injury was sufficient for the development of AKI.[178] Therefore, we sought to study the effects of formoterol on RPTC and how these effects impact formoterol-induced recovery from AKI. We hypothesized that formoterol accelerates recovery of renal function by the induction of MB in RPTC and that formoterol does so by activating a signaling pathway distinct from β_2AR agonists that do not induce MB. We proposed to test this hypothesis through three specific aims:

1. Identify differences in signaling between formoterol and clenbuterol
2. Identify the signaling pathway by which formoterol induces MB in RPTC
3. Determine the role of RPTC in the induction of MB and recovery of renal function by formoterol following AKI

CHAPTER 2

STRUCTURAL AND PHARMACOLOGICAL BASIS FOR THE INDUCTION OF MITOCHONDRIAL BIOGENESIS BY FORMOTEROL BUT NOT CLENBUTEROL

Introduction

Mitochondria play numerous roles in cellular homeostasis, including energy metabolism, synthesis of key biomolecules, regulation of reactive oxygen species, and apoptosis.[236] However, in disease states, dysfunctional mitochondria lead to metabolic defects and subsequent derangements in survival,[585] proliferation,[586] and differentiation. [261-263,587] One therapeutic strategy to treat mitochondrial dysfunction is the induction of MB.[235,588] By generating new mitochondria, MB increases cellular respiration and ATP, reduces pathologic oxidative stress, and promotes cell repair and regeneration.[247,589]

A number of signaling molecules have been shown to induce MB, including transcription factors, kinases, cyclic nucleotides, and G protein-coupled receptors (GPCRs).[588] In particular, GPCRs are attractive targets for the identification of therapeutics that induce MB because GPCRs represent numerous clinically approved receptor agonists.[590]

Previous work in our laboratory identified formoterol, a β_2AR agonist, as a potent and efficacious inducer of MB in vitro and in vivo.[567] Furthermore, in a mouse model of bilateral ischemic reperfusion-induced AKI formoterol stimulated MB with increased mitochondrial proteins and accelerated recovery of renal function.[231] Based on the success of formoterol, other β_2AR agonists were screened for induction of MB. Although several agonists were able to induce MB similar to formoterol, several other agonists, including clenbuterol, were unable to induce MB at any concentration.[566] These data suggest that a subset of biogenic β_2AR agonists modulates distinct signaling pathways from non-biogenic β_2AR agonists to induce MB.

Because both formoterol and clenbuterol, a non MB inducer, are selective β_2AR agonists,[591] we sought to identify the differences in signaling between the two agonists in primary cultures of RPTC and the signaling pathway responsible for formoterol-induced MB. Furthermore, we explored their chemical differences to identify key functional groups and structural differences that result in their differing abilities to induce MB. We found that

formoterol, but not clenbuterol, activates the Gβγ-Akt-eNOS-sGC signaling pathway and that this pathway is necessary for the transcriptional and functional changes associated with formoterol-induced MB. Molecular modeling showed that formoterol stretches further across the binding pocket than clenbuterol, allowing for simultaneous interactions with TM3, TM5, and ECL2. Additionally, the methoxyphenyl and formamide groups displayed distinct interaction fingerprints with the β_2AR that may lead to the activation of Gβγ-dependent signaling.

Results

Both formoterol and clenbuterol increase cAMP accumulation. The β_2AR couples to the stimulatory G protein $G\alpha_s$ and the inhibitory G-protein $G\alpha_i$, both of which affect the activity of adenylate cyclase and therefore the accumulation of cAMP. To assess the effects of formoterol and clenbuterol on cAMP accumulation, RPTC were co-treated for 1 h with 30 nM formoterol or 30 nM clenbuterol in the presence of 100 μM IBMX, a phosphodiesterase inhibitor to prevent cyclic nucleotide degradation. These concentrations ensure selective activation of the β_2AR while also exerting the previously observed effects on MB. Maximal cAMP accumulation occurs at 1 h (data not shown).[566] Both formoterol and clenbuterol increased cAMP relative to vehicle controls (Figure 2-1A). Because there was no difference in cAMP accumulation between the two compounds, and previous work showed that cAMP does not produce MB in RPTC, we concluded that β_2AR is functioning normally in RPTC with respect to cAMP production but that cAMP is not necessary for β_2AR-mediated MB in RPTC.

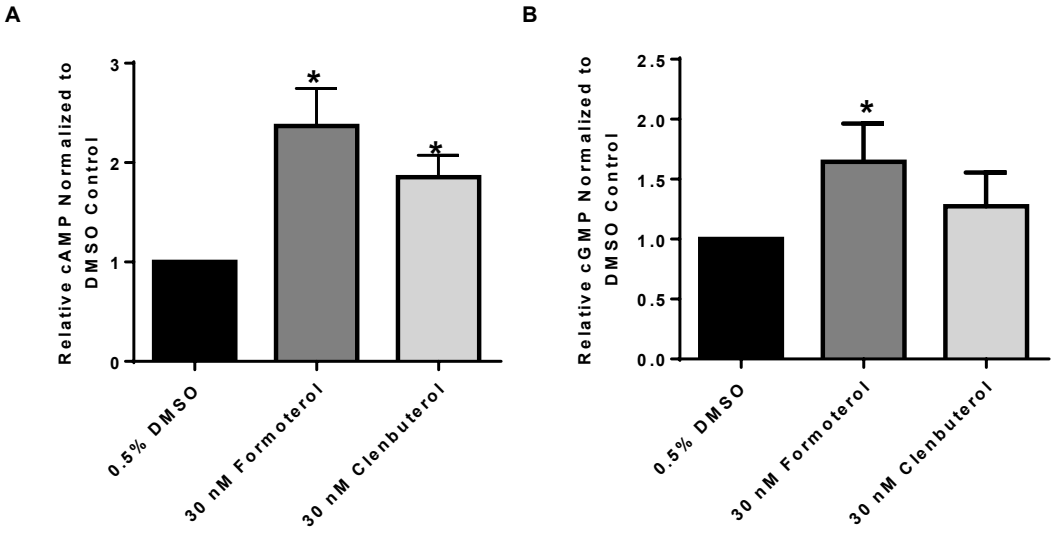


Figure 2-1. Formoterol and clenbuterol increase cAMP (A, N=3) but only formoterol increases cGMP (B, N=6) in RPTC. Levels of cAMP/cGMP were measured 1 h following treatment with formoterol or clenbuterol. Mean + SEM. *$p<0.05$, Wilcoxon signed rank test.

Formoterol, but not clenbuterol, increases Akt phosphorylation in a Gβγ-PI3K-dependent manner. In addition to their roles in the modulation of cAMP, both $G\alpha_s$ and $G\alpha_i$ release the Gβγ heterodimer. To assess the role of Gβγ, we measured Akt phosphorylation 30 min following treatment with formoterol or clenbuterol. This time point represents the earliest time point at which elevated Akt phosphorylation could be detected (data not shown). Formoterol increased Akt phosphorylation while clenbuterol did not (Figure 2-2). Pretreatment with the Gβγ inhibitor gallein[592] attenuated formoterol-induced Akt phosphorylation (Figure 2-2A), as did pretreatment with the phosphatidylinositol-4,5-biphosphate 3-kinase (PI3K) inhibitor LY294002 (Figure 2-2B).[593] These data indicate that formoterol, but not clenbuterol, increases Akt phosphorylation in a Gβγ-PI3K-dependent manner.

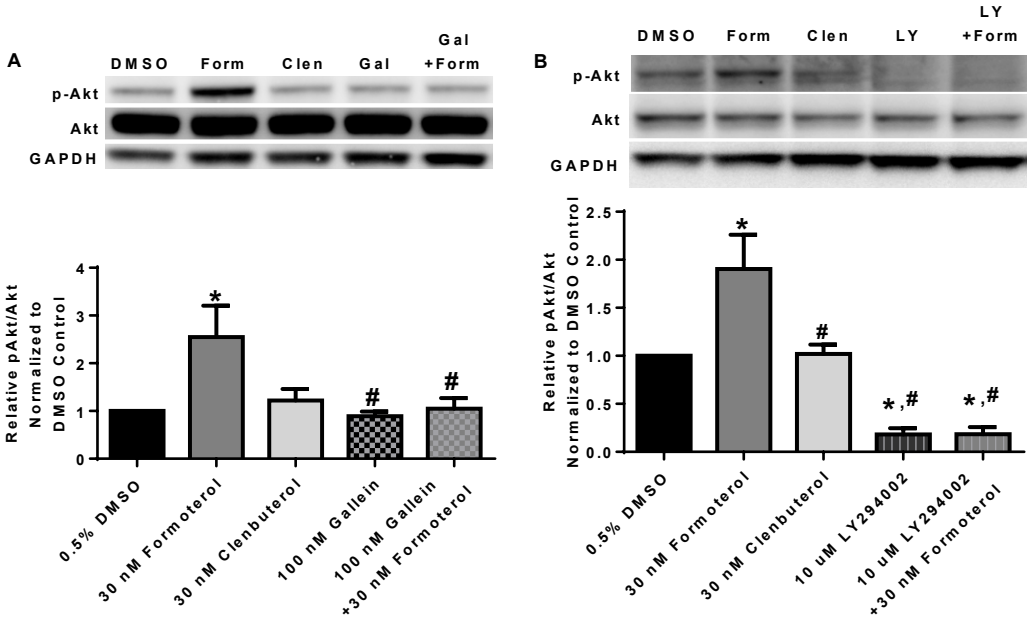


Figure 2-2. Formoterol, but not clenbuterol, activates Akt in a Gβγ-PI3K-dependent manner in RPTC. pAkt was measured following 30 min of formoterol (Form) or clenbuterol (Clen) in the presence and absence of the Gβγ inhibitor gallein (Gal)(A, N=4) or the PI3K inhibitor LY294002 (LY)(B, N=4-5). Mean + SEM. *p<0.05 vs. DMSO, #p<0.05 vs. formoterol, one-way ANOVA with Sidak's multiple comparison test.

Formoterol, but not clenbuterol, increases eNOS phosphorylation in a Gβγ-Akt-dependent manner. Among the downstream targets of Akt is endothelial nitric oxide synthase (eNOS).[594] Upon phosphorylation at S1177, eNOS is activated and increases NO generation. Because NO and NO-dependent signaling have been implicated in MB, we treated RPTC with formoterol and clenbuterol for 1 h to determine differences in eNOS phosphorylation. Formoterol increased eNOS phosphorylation relative to vehicle control, while clenbuterol did not affect eNOS phosphorylation (Figure 2-3A).

To determine the role of the Gβγ-Akt pathway in formoterol-induced eNOS phosphorylation, RPTC were pretreated with the Gβγ inhibitor gallein or the Akt inhibitor MK2206 followed by treatment with formoterol for 1 h. The allosteric Akt inhibitor MK2206 was used because it decreases Akt phosphorylation, thereby confirming that sufficient Akt inhibition had occurred.[595] Both gallein and MK2206 attenuated formoterol-induced eNOS phosphorylation (Figure 2-3B), indicating that formoterol, but not clenbuterol, activates the Gβγ-Akt-eNOS signaling pathway.

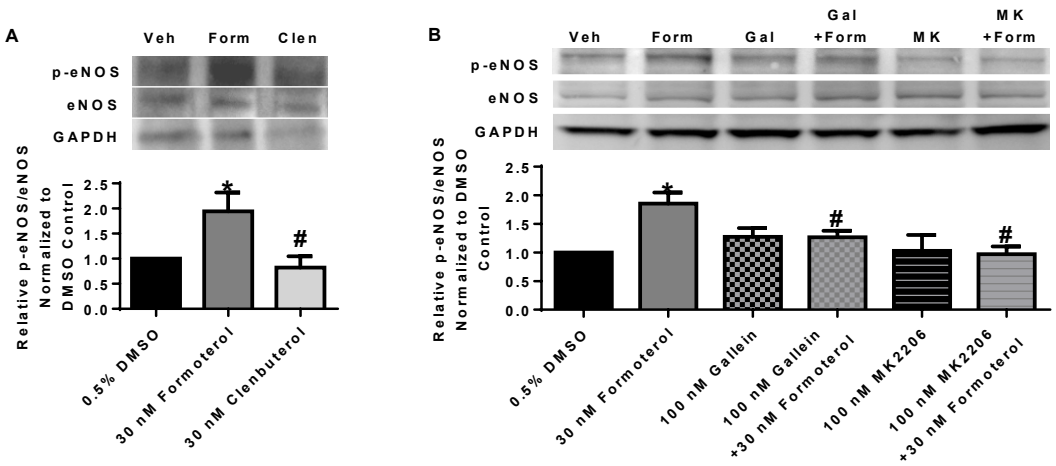


Figure 2-3. Formoterol (Form), but not clenbuterol (Clen), activates eNOS in a Gβγ- and Akt-dependent manner in RPTC. (A) p-eNOS was measured following 1 h of formoterol or clenbuterol. N=5-6. (B) Phosphorylation of eNOS was measured following 30 min of treatment with formoterol in the presence or absence of the Gβγ inhibitor gallein (Gal) or the Akt inhibitor MK2206 (MK). N=7-10. Mean + SEM. *$p<0.05$ vs. DMSO, #$p<0.05$ vs formoterol, one-way ANOVA with Sidak's multiple comparison test.

Formoterol, but not clenbuterol, increases cGMP accumulation. One of the major targets of NO is soluble guanylate cyclase (sGC). To examine any differences in cGMP accumulation, RPTC were treated with formoterol or clenbuterol in the presence of 100 μM IBMX for 1 h, and cGMP was determined by ELISA. Formoterol, but not clenbuterol, increased levels of cGMP relative to vehicle control (Figure 2-3B).

Formoterol increases maximal respiration in a Gβγ-Akt-NOS-sGC-dependent manner. Having shown that formoterol but not clenbuterol activates the Gβγ-Akt-eNOS-sGC pathway, we assessed the role of this pathway in formoterol-induced increases in FCCP-OCR, a measure of MB. RPTC were pretreated with the Gβγ-inhibitor gallein, the Akt inhibitor GDC0068, the NOS inhibitor L-NAME, and the sGC inhibitor ODQ. The orthosteric Akt inhibitor GDC0068 was used due to its greater potency and lack of isoform selectivity.[596] RPTC were then treated with formoterol or clenbuterol for 24 h, and FCCP-OCR consumption was measured. This time point was chosen based on previous studies identifying that formoterol induces MB at 24 h.[566,567] Formoterol alone increased FCCP-OCR, in agreement with previous studies (Figure 2-4A).[566,567] Pretreatment with gallein, GDC0068, L-NAME, and ODQ attenuated formoterol-induced increases in FCCP-OCR, indicating that formoterol-induced MB occurs in a Gβγ-Akt-NOS-sGC-dependent manner. Clenbuterol had no effect on FCCP-OCR.

Formoterol, but not clenbuterol, increases mRNA expression of PGC-1α and NDUFS1 in a Gβγ-Akt-NOS-sGC-dependent manner. MB requires the integrated transcription of multiple genes. To assess the effects of the Gβγ-Akt-eNOS-sGC pathway on the expression of genes associated with MB, RPTC were pretreated with gallein, GDC0068, L-NAME, and ODQ, followed by treatment with formoterol or clenbuterol for 24 h. RNA expression of PGC-1α and NADH-ubiquinone oxidoreductase core subunit S1 (NDUFS1) was assessed using RT-qPCR. Formoterol alone caused a small but significant increase in PGC-1α and NDUFS1 (Figure 2-4B, C), which was

attenuated by pretreatment with gallein, GDC0068, L-NAME, and ODQ. These data indicate that formoterol also increases transcriptional markers of MB in a Gβγ-Akt-NOS-sGC-dependent manner. Importantly, clenbuterol did not increase the mRNA expression of PGC-1α or NDUFS1. These data were further confirmed by measuring mtDNA copy number, where formoterol, but not clenbuterol, increased mtDNA copy number at 24 h (Figure 2-5).

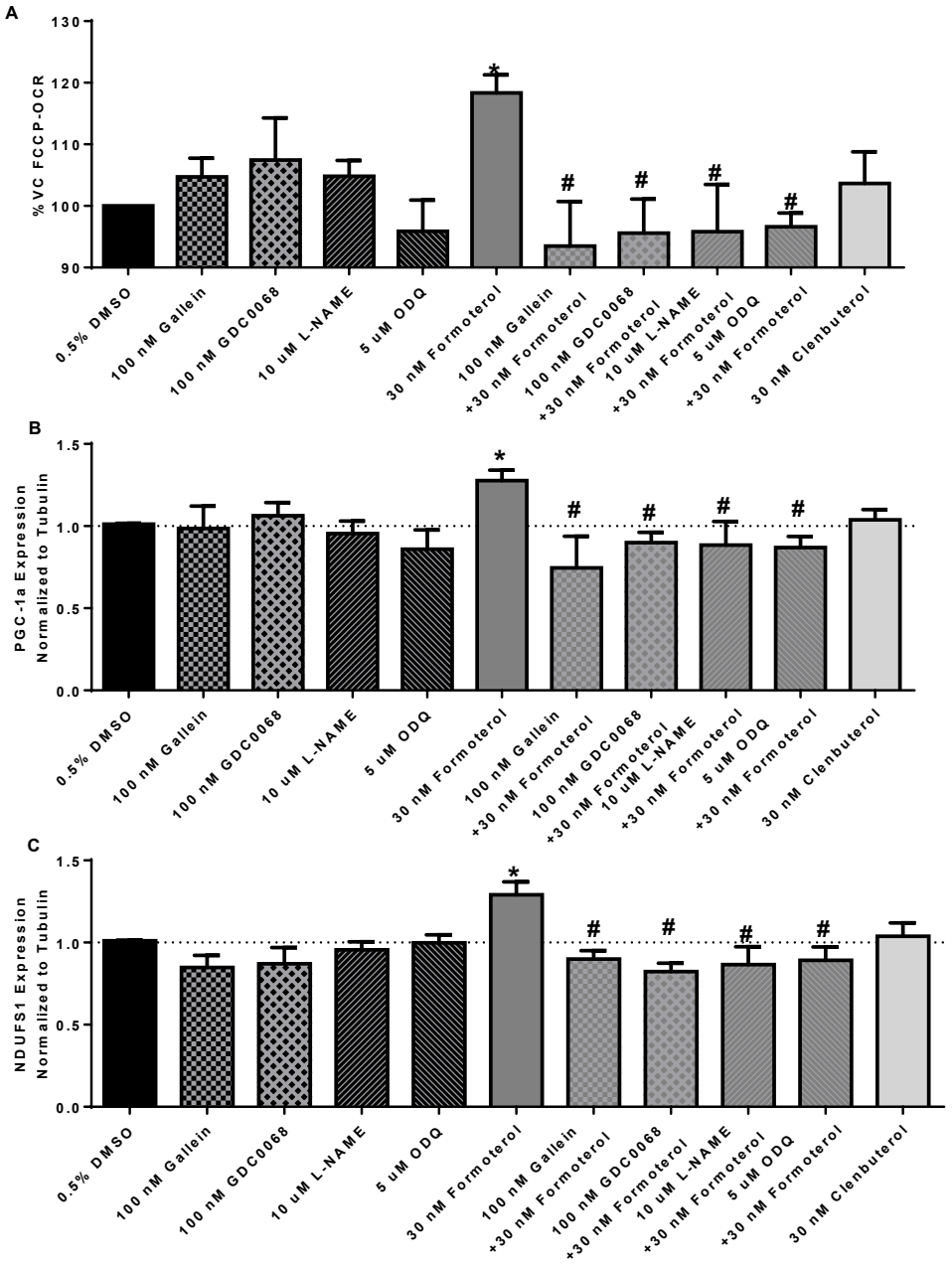


Figure 2-4. Formoterol, but not clenbuterol, induces MB in a Gβγ-Akt-NOS-sGC-dependent manner. (A) FCCP-OCR (N=3-9), (B) PGC1α mRNA (N=3-10), and (C) NDUFS1 mRNA (N=4-10) expression was measured 24 h following treatment with formoterol in the presence or absence of the Gβγ inhibitor gallein, the Akt inhibitor GDC0068, the NOS inhibitor L-NAME, and the sGC inhibitor ODQ. Mean + SEM. *p<0.05 vs DMSO, #p<0.05 vs. Formoterol, one-way ANOVA with Sidak's multiple comparison test.

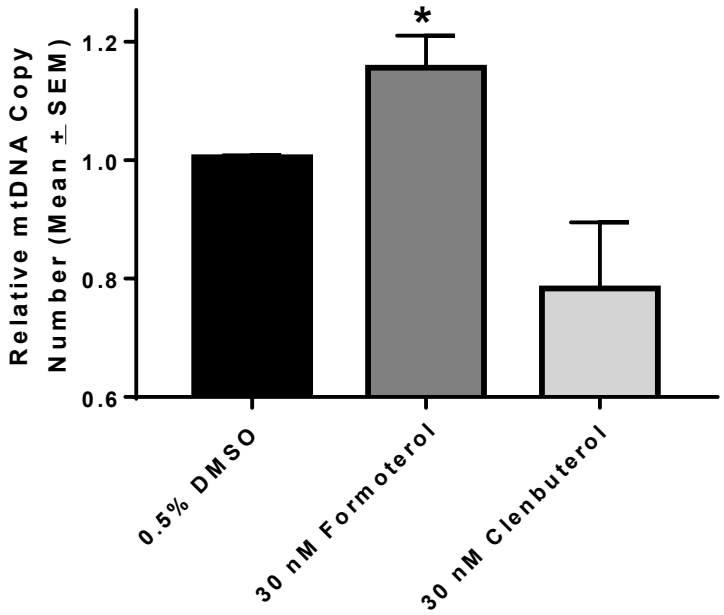


Figure 2-5. Formoterol, but not clenbuterol, increases mtDNA copy number. RPTC were treated with 30 nM formoterol or 30 nM clenbuterol for 24 h. DNA was isolated, and copy number of ND6 was measured by qPCR. N=4, *-$p<0.05$, Mann-Whitney test.

Formoterol and clenbuterol have distinct interaction fingerprints with the β_2AR. Due to the differences in signaling described above, we investigated differences in interactions of β_2AR agonists with the receptor. In addition to formoterol and clenbuterol, other ligands with known efficacy for MB were considered, including fenoterol, ritodrine, and terbutaline.[566] Ligands were docked to three inactive structures of the β_2AR (3NYA, 3NY8, 5D5B) and three active structures of the β_2AR (4LDE, 4LDL, 4LDO). Due to its longer methoxyphenyl group, formoterol was able to extend across the binding pocket to be in proximity to TM2, TM3, ECL2, and TM5 compared to clenbuterol (Figure 2-6A).

To investigate the interactions of formoterol and clenbuterol with the β_2AR with respect to distinct structural features, functional groups of the ligands were combined to form 11 structural features F1-F11 (Figure 2-7), with the catecholamine pharmacophore represented by F1-F7 and the tail group represented by F8-F11. For each conformation generated by the docking simulations, all interactions between the receptor and the ligand were tabulated. Interactions between the ligand and each receptor amino acid were separated by residue, ligand feature, and type [i.e., contact (C), arene (R), hydrogen bond donor (D), hydrogen bond acceptor (A), and ionic (I)]. Interactions at each of the 11 structural features were added for all generated conformations. To identify interactions specific to biogenic β_2AR agonists, the interactions of the compound with less efficacy for MB were subtracted from those of the compound with greater efficacy for MB (e.g., $\Sigma\text{Interactions}_{\text{Formoterol}}$-$\Sigma\text{Interactions}_{\text{Clenbuterol}}$). The resulting values were used to generate a heatmap for the interactions between the receptor and specific structural features of the ligand. Interaction pairs with more positive values (blue) indicated a greater importance for β_2AR-mediated MB (Figure 2-6B). Interaction pairs with more negative values (orange) indicate importance for stabilizing non-mitochondrially biogenic conformations of the β_2AR. This

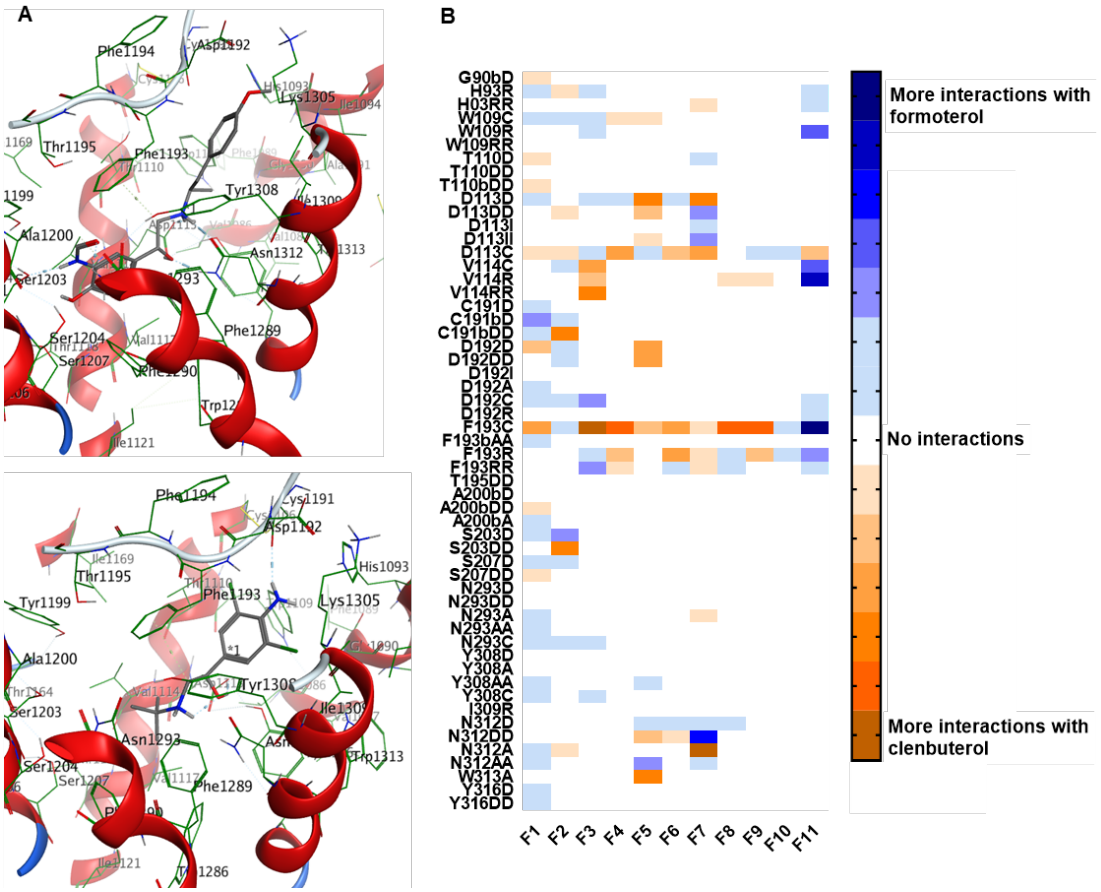
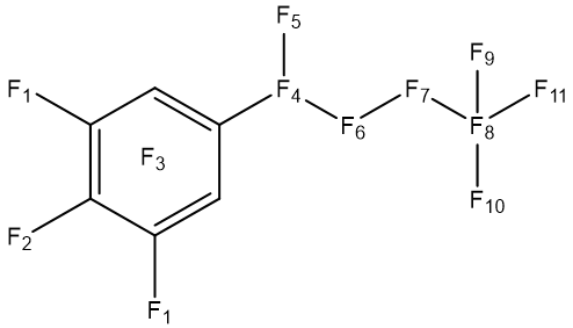


Figure 2-6. Formoterol and clenbuterol have distinct interaction fingerprints with the β_2AR. A. Representative poses of formoterol and clenbuterol in the 4LDO crystal structure. Formoterol's methoxyphenyl group (top) allows it to extend further across the binding pocket than clenbuterol (bottom), allowing formoterol to simultaneously interact with TM3, TM5, and ECL2. B. Formoterol and clenbuterol were docked to inactive structures (3NYA, 3NY8, 5D5B) and active structures (4LDE, 4LDL, 4LDO) of the β_2AR. From the top 30 poses, ligand interactions were matched with structural features F1-F11 and added for each crystal structure. More negative (orange) values correspond to a greater number of interactions with clenbuterol, while more positive (blue) values correspond to a greater number of interactions with formoterol. Interactions are displayed as Residue-interaction type (D- hydrogen bond donor, DD- strong hydrogen bond donor, I- ionic, II- strong ionic, A- hydrogen bond acceptor, AA- strong hydrogen bond acceptor, C- contact, R- arene, RR- strong arene). Residues followed by a b (e.g., G90b) indicate an interaction with the peptide backbone of the corresponding residue.



	Formoterol	Clenbuterol	(*R,R'*)-Fenoterol	(*S,R'*)-Fenoterol	Ritodrine	Terbutaline
F1	-NHCHO, -H	-Cl, -Cl	-OH, -OH	-OH, -OH	-H, -H	-OH, -OH
F2	-OH	$-NH_2$	-H	-H	-OH	-H
F3	-Ph	-Ph	-Ph	-Ph	-Ph	-Ph
F4	-CH-	-CH-	-CH-	-CH-	-CH-	-CH-
F5	-OH	-OH	-(*R*)-OH	-(*S*)-OH	-OH	-OH
F6	$-CH_2-$	$-CH_2-$	$-CH_2-$	$-CH_2-$	$-CHCH_3$	$-CH_2-$
F7	$-NH_2^+-$	$-NH_2^+-$	$-NH_2^+-$	$-NH_2^+-$	$-NH_2^+-$	$-NH_2^+-$
F8	-C-	-C-	-C-	-C-	-C-	-C-
F9	$-CH_3$	$-CH_3$	-(*R*)-CH_3	-(*R*)-CH_3	-H	$-CH_3$
F10	-H	$-CH_3$	-H	-H	-H	$-CH_3$
F11	$-CH_2$-Ph-OCH_3	$-CH_3$	$-CH_2$-Ph-OH	$-CH_2$-Ph-OH	$-CH_2$-Ph-OH	$-CH_3$

Figure 2-7. Dividing β_2AR agonists into chemical groups reveals distinct structural features. Formoterol, clenbuterol, (*R,R'*)-fenoterol, (*S,R'*)-fenoterol, ritodrine, and terbutaline were divided into 11 chemical features (F1-F11). Ph-phenyl group**.**

analysis enables the identification of features of both the ligand and the receptor that distinguish biogenic and non-biogenic β_2AR agonists.

As expected for two β_2AR agonists with a shared pharmacophore, most of the interactions showed only minor preference for formoterol or clenbuterol, with several interactions occurring with the same frequency (Figure 2-6B). Interactions between the backbone of C191 and F1 were more common for formoterol than clenbuterol, while the reverse was true for F2. Interestingly, interactions with both V114 and F193 were more common for clenbuterol over features F3-F10; however, at F11, interactions with V114 and F193 more commonly occurred with formoterol than clenbuterol. These data reveal a common pharmacophore and marked differences in the binding of formoterol and clenbuterol to the β_2AR crystal structure and suggest that interactions between distinct β_2AR residues and distinct ligand structural features must occur to activate the G$\beta\gamma$-Akt-eNOS-sGC pathway and induce MB.

To confirm the importance of the interactions identified above, we repeated the ligand interaction analysis for other ligands previously tested for MB. Fenoterol is a β_2AR agonist that induces MB in a manner similar to formoterol.[566] The (*R,R'*) enantiomer of fenoterol is a Gα_s-biased ligand; however, the (*S,R'*) enantiomer activates both Gα_s and Gα_i, potentially enabling it to activate G$\beta\gamma$-dependent signaling pathways like formoterol.[597] Comparing (*S,R'*)-fenoterol to (*R,R'*)-fenoterol showed that the former was more likely to interact with V114 and F193 at F11 and engage in hydrogen bonding interactions with S203, S207, and the backbone of C191 (Figure 2-8A). Because these differences are similar to those between formoterol and clenbuterol, these data suggest that the (*S,R'*) enantiomer of fenoterol with a greater capacity to activate G$\beta\gamma$-dependent signaling is more capable of inducing MB than the Gα_s-biased (*R,R'*) enantiomer. Comparing (*S,R'*)-fenoterol to clenbuterol showed a similar interaction profile to formoterol vs. clenbuterol (Figure 2-8B). Interactions with F193 and V114 at F11 were again more common

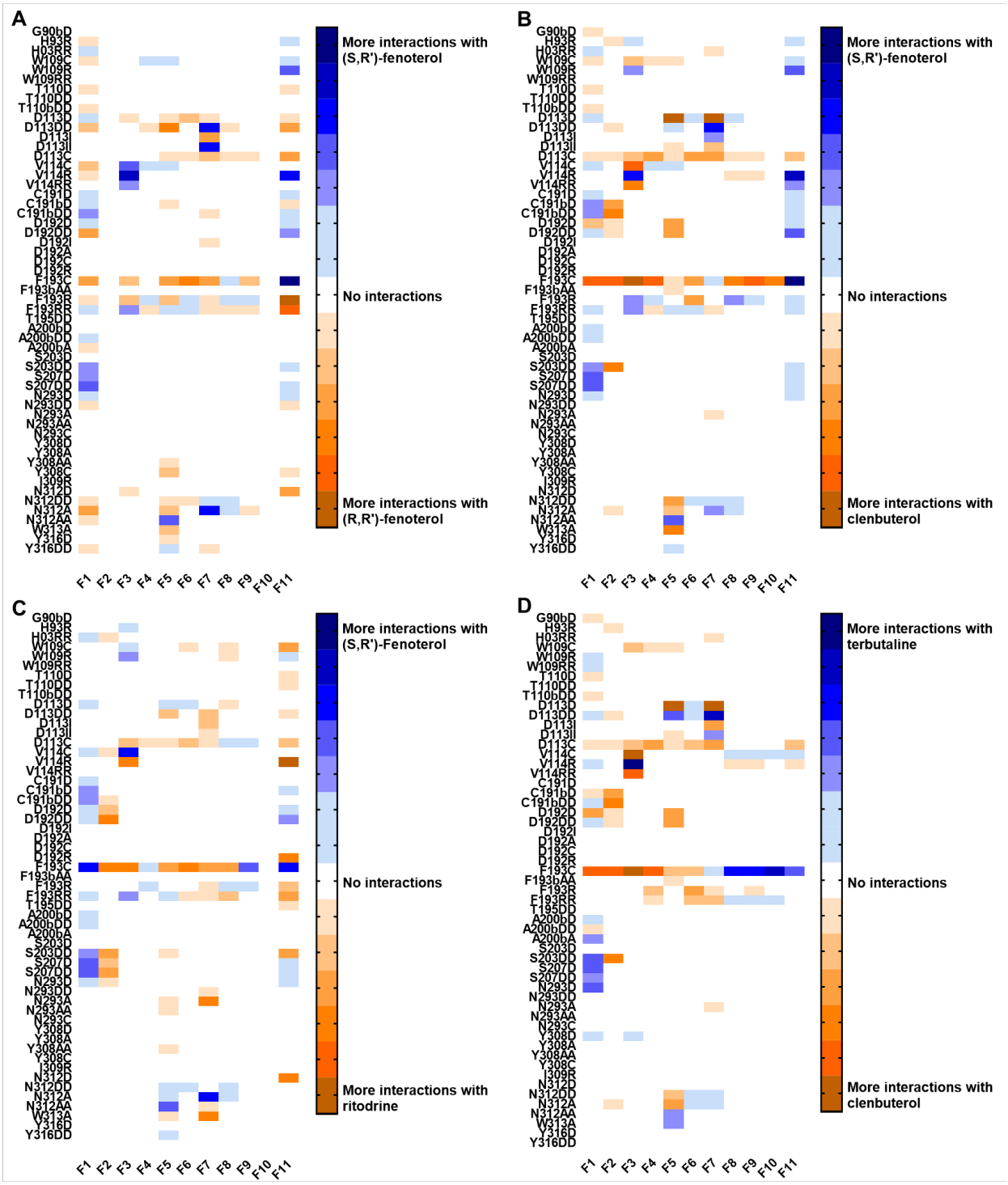


Figure 2-8. Biogenic and non-biogenic ligands have distinct interaction fingerprints with the β_2AR. (*S*,*R*')-Fenoterol, (*R*,*R*')-fenoterol, ritodrine, terbutaline, and clenbuterol were docked to inactive structures (3NYA, 3NY8, 5D5B) and active structures (4LDE, 4LDL, 4LDO) of the β_2AR. From the top 30 poses, ligand interactions were matched with structural features F1-F11 and added for each crystal structure. A. The non-G protein-biased (*S*,*R*')-fenoterol was compared to the Gα_s-biased (*R*,*R*')-fenoterol. B. The biogenic (*S*,*R*')-fenoterol was compared to the non-

biogenic clenbuterol. C. The biogenic (*S*,*R*')-fenoterol was compared to the partially biogenic ritodrine. D. The partially biogenic terbutaline was compared to the non-biogenic clenbuterol. Interactions are displayed as Residue-interaction type (D- hydrogen bond donor, DD- strong hydrogen bond donor, I- ionic, II- strong ionic, A- hydrogen bond acceptor, AA- strong hydrogen bond acceptor, C- contact, R- arene, RR- strong arene). Residues followed by a b (e.g., G90b) indicate an interaction with the peptide backbone of the corresponding residue.

with (*S,R'*)-fenoterol, although interactions with V114 had a greater tendency to be aromatic than seen with formoterol. (*S,R'*)-fenoterol also had more hydrogen bonding interactions at F1, particularly with S207, S203, and the backbone of C191.

To confirm the importance of interactions at F11, we compared (*S,R'*)-fenoterol to ritodrine (Figure 2-8C). Ritodrine can induce MB at low but not high concentrations and is structurally similar to fenoterol with identical features F3, F4, F5, F7, F8, F10, and F11 (that is, differing only at F1, F2, F6, and F9).[566] At F11, (*S,R'*)-fenoterol interacted more frequently with F193 than ritodrine. Because these ligands share a common feature F11 but differ in biogenic status, the interaction of F193 with F11 is important for β_2AR-mediated MB.

To confirm the importance of interactions at F1, we compared terbutaline to clenbuterol (Figure 2-8D). Like ritodrine, terbutaline induces MB at low but not high concentrations.[566] Terbutaline is also structurally similar to clenbuterol, sharing identical features F3-F11 (that is, differing only at F1 and F2). At feature F1, terbutaline interacted more frequently with S203, S207, and N293. When considered with the interaction profiles of formoterol and (*S,R'*)-fenoterol, these data suggest that interactions with S203 and S207 at feature F1 are important for β_2AR-mediated MB. Interestingly, terbutaline also interacted more frequently with F193 at F8, F19, F10, and F11 than clenbuterol, suggesting that the 2,5-hydroxyl substituted ring of terbutaline and fenoterol facilitates interactions with F193 at features F8-F11.

Discussion

MB plays a vital role in regulating cellular metabolism, differentiation, and repair, and its pharmacologic induction has great therapeutic potential in a variety of disease states.[235,588] Here, we show that in RPTC, formoterol, but not clenbuterol, activates the Gβγ-Akt-eNOS-sGC signaling pathway and that this pathway is necessary for formoterol-induced MB (Figure 2-9). Importantly, these experiments were performed in metabolically competent primary cells that can better mirror

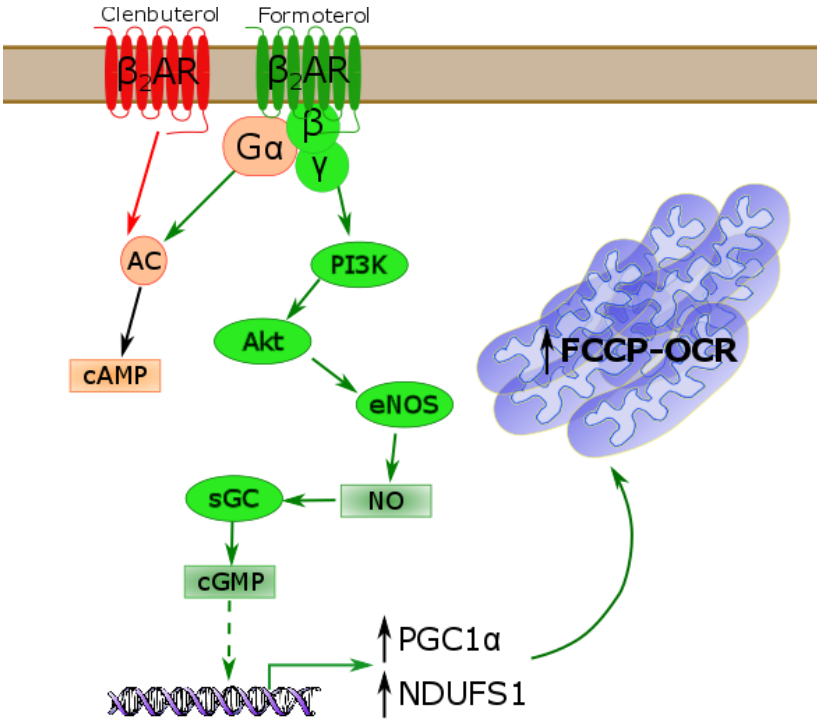


Figure 2-9. Formoterol, but not clenbuterol, induces MB in a Gβγ-Akt-NOS-sGC-dependent manner despite increased cAMP accumulation. Both formoterol and clenbuterol activate $G\alpha_s$-dependent signaling to activate adenylate cyclase (AC) and promote cAMP accumulation. However, only formoterol activates the Gβγ-PI3K-Akt-eNOS-sGC pathway, and it is this pathway that is necessary for β2AR-induced MB in RPTC.

in vivo signaling and metabolism compared to immortalized cell lines. Therefore, these data suggest a novel role of Gβγ-dependent signaling for GPCR-mediated MB in other non-renal tissues.

The Gβγ heterodimer is released from heterotrimeric G proteins following GPCR activation. Gβγ is primarily activated by the $G_{i/o}$ family of G proteins; however, other G protein families, including G_s, release Gβγ.[598] Gβγ heterodimers have varied effects on signal transduction, including PI3K activation, adenylate cyclase stimulation, adenylate cyclase inhibition, MAPK activation, and GRK activation, depending on their constituent Gβ and Gγ subunits.[599] GPR43 was shown to signal through Gβγ and its activation by acetate induced MB,[600] but the lack of inhibitor studies meant that a causal link between Gβγ and MB was not established. By pretreating cells with the Gβγ inhibitor gallein, we identified that Gβγ-dependent signaling is a key pathway for GPCR-mediated MB.

It is important to note that the present study does not determine whether or not Gβγ directly activates the PI3K-Akt pathway. In addition to the activation of the Ras-PI3K-Akt pathway, Gβγ can also facilitate GRK2 recruitment to the receptor and lead to arrestin-dependent signaling. Arrestins are also capable of activating Akt in a PI3K-Src-dependent mechanism.[601] However, given that the arrestin-biased agonist isoetharine is unable to induce MB,[566] this mechanism is unlikely.

Prolonged activation of Akt enhances cellular survival but can lead to a decrease in mitochondrial function.[602-604] In contrast, acute activation of Akt is responsible for the effects of multiple inducers of MB, including (-)-epicatechin and erythropoietin.[326,605] For such compounds, the role of Akt seems to be limited to the phosphorylation of eNOS, leading to NO generation. Nitric oxide is a potent inducer of MB in vitro and in vivo through the activation of sGC and subsequent cGMP accumulation.[606] However, because NO is a free radical, sustained NOS

activation can increase oxidative and proteotoxic stress and can inhibit complex I of the electron transport chain.[607,608] Thus, both the signaling cascade activated by a compound and the duration of that signaling contribute to the therapeutic potential of inducers of MB.

Interestingly, there are conflicting data regarding the role of Gβγ in acute organ injury, which is frequently characterized by mitochondrial dysfunction. Inhibition of Gβγ by gallein inhibits RPTC proliferation and thereby exacerbates ischemic AKI.[609] In contrast, gallein also prevents inflammatory cell infiltration,[592] leading to enhanced recovery following ischemic AKI.[610] Although neither study assessed mitochondrial activity, both regeneration following injury and inflammatory cell chemotaxis are enhanced by increases in mitochondrial activity.[230,611]

The β_2AR is a prototypical class A GPCR and has been extensively studied for its role in cellular signaling and the structural features that enable such signaling. This study is the first to examine the receptor-ligand interactions that distinguish mitochondrial biogenic β_2AR agonists from non-biogenic β_2AR agonists. As expected for two agonists of the β_2AR, formoterol and clenbuterol have a common pharmacophore, and many of the interactions showed little preference for formoterol or clenbuterol. Nonetheless, the structural dissimilarities of the two compounds led to several distinct receptor-ligand interactions. In particular, interactions with V114 and F193 tended to occur more frequently at the methoxyphenyl group on formoterol, while C191 and its peptide backbone interacted more frequently with the formamide group of formoterol. To enable these interactions to occur, formoterol binds to the β_2AR in a conformation that places it near the deeper pocket residues of TM3 and TM5 as well as near the shallower residues of TM2, ECL2, and ECL4. Our observation of the proximity to ECL2 and ECL4 with formoterol is in agreement with NMR studies showing that formoterol weakens the ionic interaction between D192 and K305.[612] Furthermore, ECL2 flexibility is important to ligand activity at the β_2AR and for other GPCRs, such as the D_2 dopamine receptor.[613]

Modeling interactions of other β_2AR agonists with the receptor further supported the role of a subset of receptor ligand interactions in stabilizing conformations of the β_2AR that lead to MB. When compared to the non-biogenic agonist clenbuterol or the partial agonist of MB ritodrine, the hydroxyphenyl group (F11) of (*S,R'*)-fenoterol was more likely to engage in contact interactions with F193 and hydrogen bond donor interactions with C191 and its backbone. Additionally, its 3,5-hydroxyl groups (F1) were more likely to act as hydrogen bond donors for S203 and S207. These results are in agreement with previous docking studies of fenoterol with the β_2AR.[614] Similarly, when compared to clenbuterol, the partial agonist of MB terbutaline had more contact interactions between its *tert*-butyl group (F8-F11) and F193 and hydrogen bond donor interactions between its 3,5-hydroxyl groups (F1) and S203, S207, and N293. Previous docking studies with terbutaline identified interactions between terbutaline and F193 but not between S203, S207, or N293.[615] However, those studies generated homology models of the rat β_2AR from 2rh1, which represents an inactive conformation of the receptor,[616] while our study employed both active and inactive structures of the β_2AR.

Previous NMR studies have shown that formoterol and clenbuterol engage different conformations of the β_2AR,[617] particularly regarding the conformational shift of TM6. Indeed, numerous studies using NMR and mass spectrometry have shown that functionally similar agonists can effect distinct active conformations of the receptor.[618-620] Additionally, the conformational flexibility of the β_2AR allows for multiple "active" conformations that may lead to differences in effector coupling. Among its active conformations, formoterol may stabilize a set of biogenic β_2AR conformations that are thermodynamically unfavorable for clenbuterol.

In conclusion, this study identified a distinct signaling pathway activated by the mitochondrial biogenic β_2AR agonist formoterol but not by the non-biogenic β_2AR agonist clenbuterol in metabolically competent primary cells. This G$\beta\gamma$-Akt-eNOS-sGC pathway is

necessary for the transcriptional and functional changes associated with MB. We also identified distinct structural features and ligand interactions that may allow formoterol to activate this pathway. Together, these data can facilitate the development of novel β_2AR agonists that selectively stimulate the G$\beta\gamma$-Akt-eNOS-sGC pathway to induce MB and recovery from acute and chronic degenerative diseases.

Methods

Reagents. Anti-GAPDH antibody was purchased from Fitzgerald Antibodies (Acton, MA) and was used at a dilution of 1:1,000. Anti-phospho-Akt (Ser473), anti-Akt, and anti-phospho-eNOS (Ser1177) antibodies were purchased from Cell Signaling (Danvers, MA) and were all used at a dilution of 1:1,000. Anti-eNOS antibody (1:500 dilution), anti-mouse IgG (1:10,000 dilution), and anti-rabbit IgG (1:2,000 dilution) antibodies were purchased from Abcam (Cambridge, MA). MK2206 and GDC0068 were purchased from SelleckChem (Houston, TX). Gallein, LY294002, L-NAME, and ODQ were purchased from Tocris (Ellisville, MO). All other chemicals were purchased from Sigma (St. Louis, MO).

Isolation and culture of proximal tubule cells. Female New Zealand white rabbits (1.5-2.0 kg) were purchased from Charles River Laboratories (Wilmington, MA). RPTCs were isolated via the iron oxide perfusion method, and RPTCs were cultured under improved conditions as described previously[621,622]. Three days after initial playing, dedifferentiated RPTCs were trypsinized and replated on XF-96 polystyrene culture microplates (Seahorse Bioscience, North Billerica, MA) at a density of 18,000 cells/well and were maintained at 37ºC for 3 days before pharmacological manipulation. For other RPTC experiments, isolated renal proximal tubules were plated in 35 mm dishes and used at confluence 6 days after initial plating. All experiments were carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All procedures were approved by the Institutional Animal

Care and Use Committees of the Medical University of South Carolina and the University of Arizona and appropriate efforts were made to reduce animal suffering.

Measurement of oxygen consumption. The oxygen consumption rate (OCR) of RPTCs was measured using the Seahorse Bioscience XF-96 Extracellular Flux Analyzer as previously described.[623] RPTCs in 96-well assay plates were treated with vehicle control (dimethylsulfoxide (DMSO), <0.5%) or with experimental compounds. Basal OCR was measured, followed by injection of 10 μM carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) to allow for the measurement of uncoupled OCR (FCCP-OCR), a marker of MB.

Protein isolation and immunoblotting. Freshly isolated RPTCs were suspended in protein lysis buffer (1% Triton X-100, 150 mM NaCl, and 10 mM Tris-HCl, pH 7.4; 1 mM EDTA; 1 mM EGTA; 2 mM sodium orthovanadate; 0.2 mM phenylmethylsulfonyl fluoride; 1 mM HEPES, pH 7.6) containing protease inhibitors and phosphatase inhibitors (Sigma-Aldrich, St. Louis, MO). Following sonication, protein was quantified using a bicinchoninic acid assay, subjected to SDS-PAGE, transferred onto nitrocellulose membranes, and incubated with primary and secondary antibodies. Membranes were detected using chemiluminescence and processed using ImageJ (NIH, Bethesda, MD) software.

Nucleic acid isolation and quantitative polymerase chain reaction. To measure RNA expression, RPTC were scraped in TRIzol (Life Technologies, Grand Island, NY), and RNA was isolated using a phenol-based centrifugation method. cDNA was reversed transcribed from 5 μg RNA using the iScript Advanced cDNA Synthesis Kit (BioRad, Hercules, CA), diluted 1:10, and 5 μL added to a real-time SYBR green quantitative polymerase chain reaction master mix (BioRad). Changes in gene expression were calculated based on the Δ-Δ threshold cycle method. The following primers were used: PGC1α forward (AGGAAATCCGAGCCGAGCTGA), PGC1α reverse (GCAAGACGGAGACACATCAAA), NDUFS1 forward (AGATGATTTGGGAACAACAG), NDUFS1 reverse (TAGGGCTTAGAGGTTAGAGC),

tubulin forward (CTCTCTGTCGATTACGGCAAG), and tubulin reverse (TGGTGAGGATGGAGTTGTAGG).

To measure mtDNA copy number, RPTC were scraped in phosphate buffered saline, and DNA was extracted using the DNeasy Blood and Tissue kit (QIAGEN, Valencia, CA). PCR products were amplified from 50 ng of cellular DNA using a real-time SYBR green quantitative polymerase chain reaction master mix (BioRad). For estimation of mtDNA, the NADH dehydrogenase subunit 6 (ND6) gene was used and normalized to tubulin. The following primers were used: ND6 forward (ACTGCGATGGCAACTGAGGAGTAT), ND6 reverse (ACCATAACTATACAACGCCGCCAC), tubulin forward (CTCTCTGTCGATTACGGCAAG), and tubulin reverse (TGGTGAGGATGGAGTTGTAGG).

Measurement of cyclic nucleotides. RPTCs in 35-mm dishes were treated with vehicle control or the compound of interest for 1 h. RPTCs were then harvested according to the manufacturer's protocol (Cayman Chemical, Ann Arbor, MI). Levels of cAMP and cGMP were measured using a commercially available enzyme-linked immunosorbent assay kit. Values were normalized to protein as quantified by a bicinchoninic acid assay followed by normalization to vehicle control for each biological replicate.

Molecular modeling. Modeling, simulations, and visualizations were performed using MOE (Molecular Operating Environment) version 2015.1001 (Chemical Computing Group). The structural files used as input for analysis and docking simulations were PDB codes 3NYA, 3NY8, 4LDE, 4LDL, 4LDO, and 5D5B. Before analysis and simulations, all atoms and molecules other than the receptor and the ligand were removed. The receptor and all ligands were protonated at pH 7.4 in MOE. Initial placement calculated 30 poses per molecule using triangle matching placement with London dG scoring. All 30 poses were then refined using induced fit with London dG scoring. Ligand interactions were assessed using the protein-ligand interaction fingerprint (PLIF) function in MOE. To score interaction frequencies, interactions with individual atoms

were tabulated. Interactions with the active ligand formoterol were assigned a value of 1, while interactions with the inactive ligand clenbuterol were assigned a value of -1, and interactions for all poses across all crystal structures were added to generate overall ligand interaction frequencies at structural features.

Statistical analysis. Data are expressed as means $\pm$ S.E.M. ($N \geq 3$) for all experiments. Each N represents a biological replicate. Multiple comparisons of normally distributed data were analyzed by one-way analysis of variance, as appropriate. Single comparisons were analyzed with the Wilcoxon signed rank test where appropriate. The criterion for statistical differences was $p<0.05$ for all comparisons.

CHAPTER 3

PROXIMAL TUBULE CELL DELETION OF THE β_2 ADRENERGIC RECEPTOR PREVENTS FORMOTEROL-INDUCED RECOVERY OF MITOCHONDRIAL AND RENAL FUNCTION AFTER ISCHEMIA-REPERFUSION INJURY

Introduction

AKI is a rapid loss of renal function that occurs in over 20% of hospitalized patients and has a mortality rate of 25%.[2,3] AKI has numerous causes, including hypotension, nephrotoxic drug administration, and renal ischemia-reperfusion injury (IRI).[5] Unfortunately, treatment of AKI remains limited to supportive care and renal replacement therapy.

The difficulty in treating AKI is the numerous cell types involved, including immune cells,[139] endothelial cells,[624] and the renal epithelium.[113] RPTC are a highly oxidative and regenerative cell type that plays a central role in the pathogenesis of AKI.[176,177] RPTC exhibit mitochondrial fragmentation and dysfunction with persistent suppression of MB after AKI.[203,205,207] Transgenic mouse models have shown that decreased MB worsens AKI, while increased MB accelerates recovery.[625]

Drugs that increase MB accelerate recovery from AKI with concomitant rescue of mitochondrial protein expression and function.[567] One such drug is the FDA-approved β_2AR agonist formoterol.[567] We reported that formoterol treatment restored renal function with concomitant increases in mitochondrial protein expression and function after AKI in mice.[231] Recently, we elucidated the mechanism of formoterol-induced MB in RPTC.[626] Formoterol binding to the β_2AR results in the release of G$\beta\gamma$ heterodimer, the activation of Akt, the phosphorylation of eNOS, and increased soluble guanylyl cyclase activity and cGMP. This pathway increased PGC-1α, the master regulator of MB,[232] with concomitant induction of MB.

Because the β_2AR is ubiquitously expressed (e.g. T cells, macrophages, neutrophils, endothelial cells and RPTC), it is not clear which cell(s) are responsible for formoterol induced MB and recovery of renal function following IR-induced AKI. The goal of this study was to determine the specific role of RPTC β_2AR in AKI and formoterol-induced recovery of mitochondrial and renal function using a mouse with proximal tubule specific deletion of the β_2AR.[627]

Results and Discussion

RPTC-specific deletion of the β_2AR in mice was achieved by breeding γGT-Cre mice with ADRB2$^{Flox/Flox}$ (WT) mice to create a γGT-Cre:ADRB2$^{Flox/Flox}$ mouse (KO) (Figure 3-1A). To assess expression of the β_2AR in RPTC in these mice, renal cortical ADRB2 DNA and mRNA were measured using qPCR. Consistent with loss of β_2AR in RPTC, KO mice had an 80% reduction in renal cortical ADRB2 DNA and mRNA expression and increased γGT-Cre DNA expression relative to WT mice (Figure 3-1B-D).

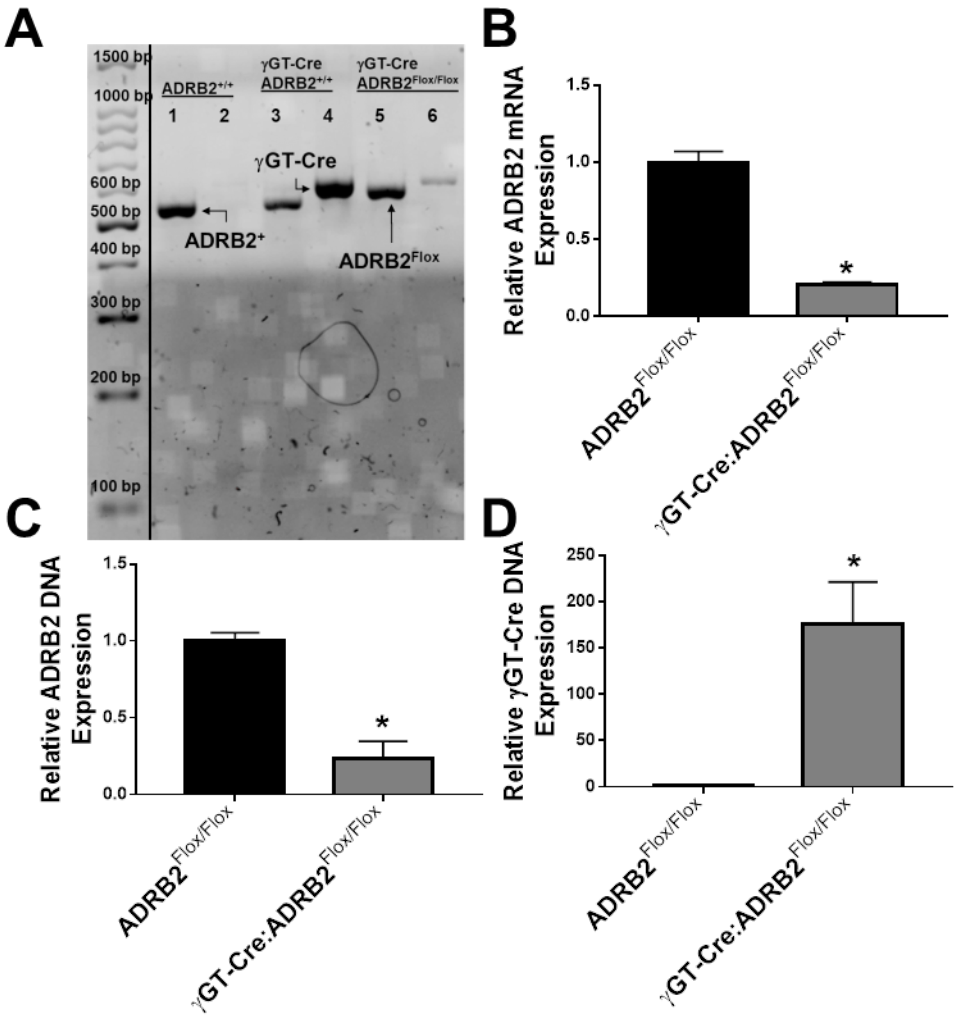


Figure 3-1. γGT-Cre:ADRB2$^{Flox/Flox}$ mice have proximal tubule specific deletion of the β_2 adrenergic receptor. A. DNA electrophoresis of: an ADRB2$^{+/+}$ mouse homozygous for ADRB2^{+} (lane 1) and not expressing gGT-Cre (lane 2); a mouse γGT-Cre:ADRB2$^{+/+}$ homozygous for ADRB2^{+} (lane 3) and expressing gGT-Cre (lane 4); and a γGT-Cre:ADRB2$^{Flox/Flox}$ mouse homozygous for ADRB2^{Flox} (lane 5) and expressing gGT-Cre (lane 6). B. RT-PCR of ADRB2 mRNA in ADRB2$^{Flox/Flox}$ and γGT-Cre:ADRB2$^{Flox/Flox}$ mice. C, D. PCR of ADRB2 (C) and γGT-Cre (D) DNA. All samples are from renal cortex. Mean±SEM. N=4-5, *-p<0.05, Student's T-test.

The role of RPTC β_2AR on recovery from AKI was determined in KO and WT mice subjected to renal IRI followed by treatment with vehicle or formoterol (0.3 mg/kg, i.p) after 24 h and then daily for 144 h. WT and KO mice had similar increases in SCr at 24 h, indicating no difference in initial injury (Figure 3-2A). As previously described,[231] WT mice treated with formoterol exhibited recovery from AKI at 144 h as measured by decreases in SCr and renal cortical KIM-1 protein (Figure 3-2B, E). In contrast, KO mice treated with formoterol did not exhibit decreases in SCr and renal cortical KIM-1 at 144 h. These findings were confirmed by histopathology in that formoterol-treated WT mice had less necrosis than vehicle-treated animals, while formoterol failed to decrease necrosis in KO mice at 144 h (Figure 3-2C, D). Together, these findings provide evidence that following AKI, formoterol exerts its effects on renal recovery by activating the RPTC β_2AR.

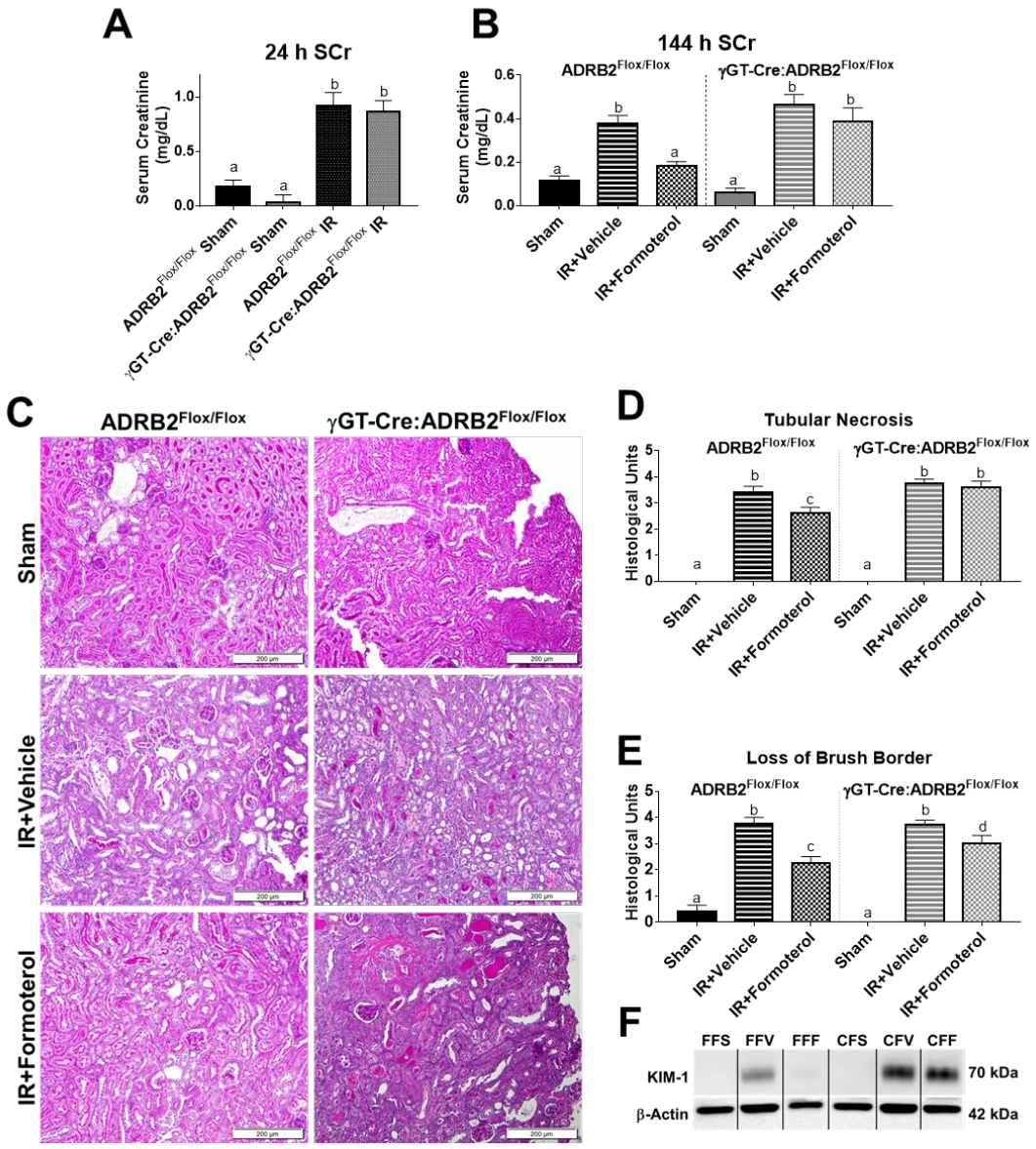


Figure 3-2. Proximal tubule deletion of the β_2 adrenergic receptor blocks the effects of formoterol on renal function following AKI. ADRB2$^{Flox/Flox}$ and γGT-Cre:ADRB2$^{Flox/Flox}$ mice were subjected to sham or renal IRI surgery. Mice were treated with 0.3% DMSO (Vehicle) or 0.3 mg/kg formoterol (Formoterol) once daily beginning at 24 h and were euthanized at 144 h. A, B.) Serum creatinine at 24 h (A) and 144 h (B) following IR. C.) PAS stained kidney sections. D,E.) Semi-quantitative scoring of tubular necrosis (D). E.) Representative blot of renal cortical KIM-1 at 144 h following IRI. FFS-ADRB2$^{Flox/Flox}$ sham, FFV-ADRB2$^{Flox/Flox}$ IR+0.3% DMSO, FFF-ADRB2$^{Flox/Flox}$ IR+0.3 mg/kg formoterol, CFS-γGT-Cre:ADRB2$^{Flox/Flox}$ Sham, CFV- γGT-Cre:ADRB2$^{Flox/Flox}$ IR+0.3% DMSO, CFF- γGT-Cre:ADRB2$^{Flox/Flox}$ IR+Formoterol. Mean±SEM. N=4-9. Different letters denote $p<0.05$, Two-Way ANOVA with Fisher's LSD test.

RPTC have high mitochondrial content to maintain proper solute transport across the tubular lumen.[112,175,176] Following AKI, MB is persistently suppressed and recovery of mitochondrial content is associated with recovery of renal function and improved outcomes.[205] The effects of RPTC β_2AR on mitochondrial content were assessed by measuring mtDNA and mitochondrial proteins using qPCR and immunoblot analysis, respectively. Formoterol restored mtDNA copy number in WT but not KO mice after IRI (Figure 3-3A). Similarly, KO mice subjected to IRI and treated with formoterol demonstrated no recovery of nuclear-encoded NDUFS1 and the mitochondrial-encoded COX1, electron transport chain (ETC) proteins and markers of MB (Figure 3-3B). Thus, activation of RPTC β_2AR by formoterol rescues markers of MB following AKI. Interestingly, KO shams had elevated expression of NDUFS1, which suggests that the β_2AR may regulate mitochondrial homeostasis in healthy RPTC.

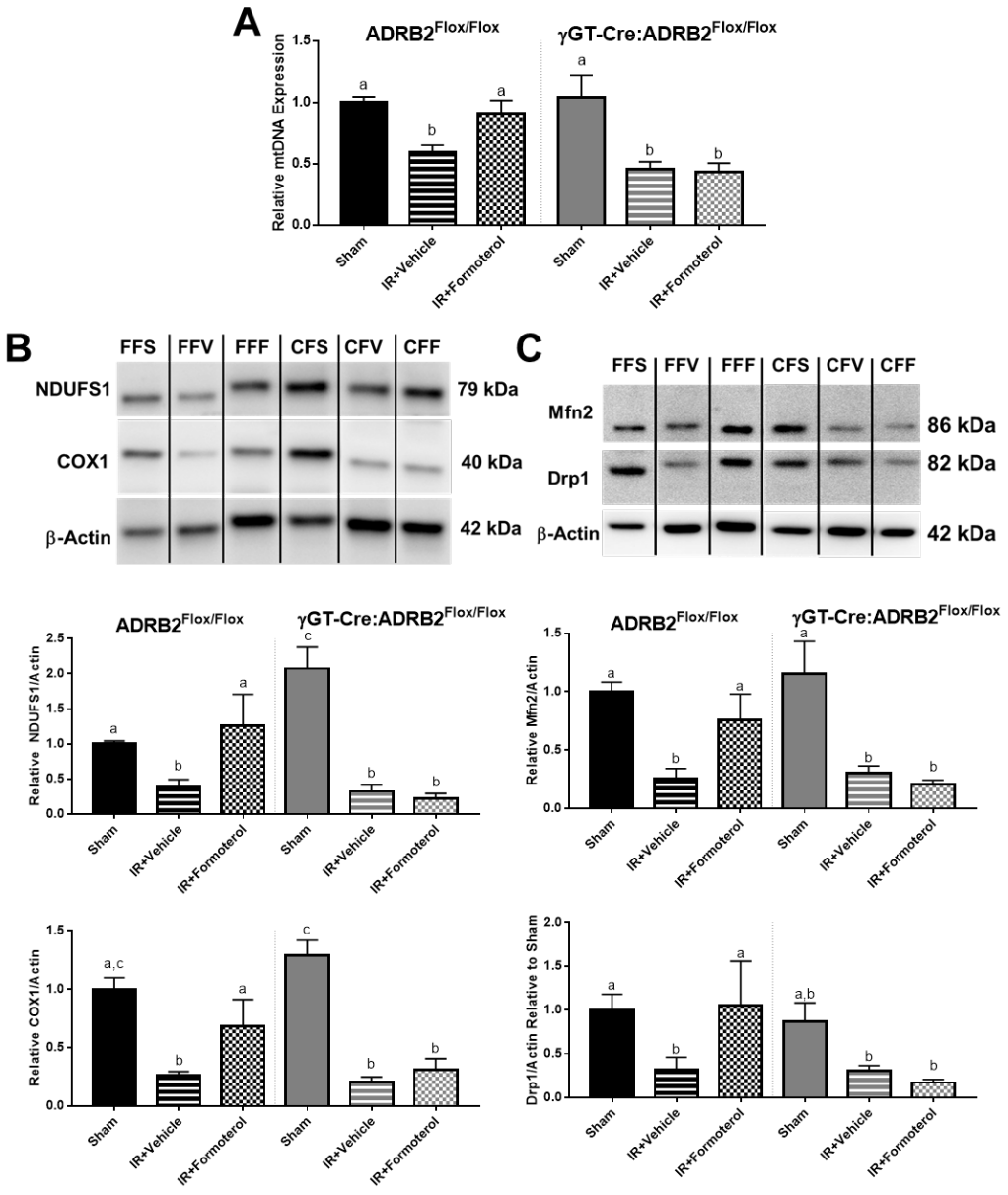


Figure 3-3. Proximal tubule cell β_2 adrenergic receptor mediates formoterol-induced rescue of mitochondrial homeostasis following IRI-AKI. A.) Mitochondrial DNA copy number. B.) Representative blots and quantification of nuclear-encoded (NDUFS1) and mitochondrial-encoded (COX1) proteins in renal cortex 144 h after IRI. C.) Representative blots and quantification of mitochondrial dynamic proteins Mfn2 and Drp1 in renal cortex 144 h after IRI. FFS-ADRB2$^{Flox/Flox}$ sham, FFV-ADRB2$^{Flox/Flox}$ IR+0.3% DMSO, FFF-ADRB2$^{Flox/Flox}$ IR+0.3 mg/kg formoterol, CFS-γGT-Cre:ADRB2$^{Flox/Flox}$ sham, CFV- γGT-Cre:ADRB2$^{Flox/Flox}$ IR+0.3% DMSO, CFF- γGT-Cre:ADRB2$^{Flox/Flox}$ IR+0.3 mg/kg Formoterol. Mean±SEM. N=4-9. Different letters denote $p<0.05$, Two-Way ANOVA with Fisher's LSD test.

To further understand mitochondrial changes under these experimental conditions in the absence and presence of formoterol, electron micrographs were obtained and mitochondrial number and morphology were quantified using ImageJ and the Trainable Weka Segmentation plugin. In WT mice, renal cortical mitochondrial number and total mitochondria area decreased after IRI and formoterol restored these parameters, indicating that formoterol induced MB (Figure 3-4A-C). Sham-operated KO mice had fewer mitochondria, providing evidence that RPTC β_2AR regulates mitochondrial homeostasis under physiological conditions.

In addition to the restoration of ETC proteins, formoterol restored mitochondrial fission and fusion proteins Drp1 and Mfn2, respectively, in WT but not KO mice (Figure 3-3C). Because Drp1 and Mfn2 are regulated by the PGC-1α,[246,628,629] and expression of both proteins is restored following formoterol-treatment in WT mice, the recovery of Mfn2 and Drp1 is linked to formoterol-induced activation of PGC-1α. Drp1 is thought to be detrimental following injury by enhancing mitochondrial fragmentation, reactive oxygen species production, and apoptosis.[225] Following AKI, mitochondrial fragmentation is increased in RPTC in a Drp1-dependent manner [203], and decreased Mfn2 expression potentiates this fragmentation.[227,630] As such, formoterol activation of β_2AR and restoration of Drp1 and Mfn2 expression may improve mitochondrial dynamics and contribute to recovery of mitochondrial function after IRI by affecting mitochondrial dynamics.

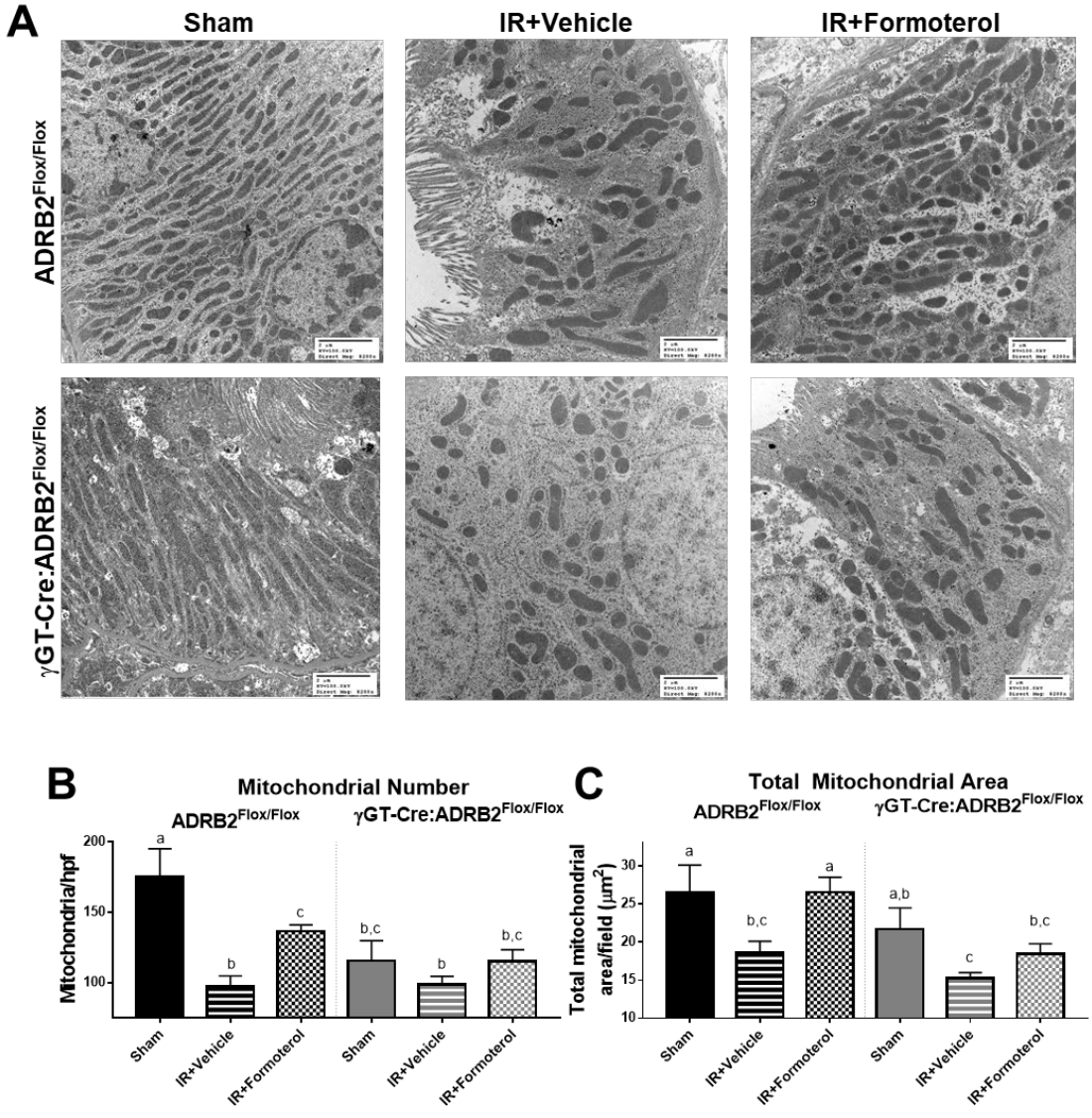


Figure 3-4. The role of proximal tubule-cell β_2 adrenergic receptor on mitochondrial content and morphology in renal cortex. A.) Representative electron micrographs of ADRB2$^{Flox/Flox}$ and γGT-Cre:ADRB2$^{Flox/Flox}$ subjected to sham or IRI surgery followed by treatment with 0.3% DMSO or 0.3 mg/kg formoterol daily for 144 h. B.) Quantification of mitochondria per field. C.) Quantification of total mitochondrial area per field. All images were acquired at x8,200 magnification with at least 5 fields per animal. N=3-8. Mean±SEM. Different letters indicate $p<0.05$, Two-Way ANOVA with Fisher's LSD test.

Proper balance of MB, fission, and fusion is important for recovery from AKI. Deletion of PGC-1α, a key transcriptional regulator of MB, worsens RPTC injury while its overexpression promotes MB to accelerate recovery.[205,625] Pharmacologic induction of MB accelerates recovery of mitochondrial and renal function following AKI.[231,631,632] Formoterol-treated mice lacking the β_2AR in RPTC failed to recover mtDNA copy number, mitochondrial protein expression, and mitochondrial number and area following AKI. In addition, sham-operated KO mice had fewer total mitochondria without a decrease in mtDNA copy number and elevated NDUFS1 protein expression. These data provide evidence that the β_2AR plays a role in RPTC mitochondrial homeostasis in healthy mice. While previous studies have shown that formoterol increases mRNA, protein, and functional markers of mitochondria in the kidney,[231,567] this study shows for the first time that formoterol induces *bona fide* MB in RPTC. In summary, these data underscore the importance of RPTC mitochondria as a therapeutic target, that β_2AR regulates renal mitochondrial homeostasis, and that GPCR ligands such as formoterol can induce MB to accelerate recovery from renal function.

Methods:

Animal Use. ADRB2$^{Flox/Flox}$ mice were mated with γGT-Cre mice to obtain γGT-Cre:ADRB2$^{Flox/Flox}$ mice. Eight- to ten-week-old mice were subjected to bilateral renal ischemia-reperfusion injury as previously described.[207] Dosing was initiated 24 h after reperfusion, and mice were given a daily injection of 0.3 mg/kg formoterol fumarate dihydrate (Sigma-Aldrich F9552) or vehicle (0.3% DMSO in normal saline) *via* intraperitoneal injection. Blood was collected by retro-orbital bleeding puncture and serum creatinine (SCr) was determined using the Creatinine Enzymatic Reagent Assay kit (Diazyme) according to manufacturer's protocol. All experiments were carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All procedures were

approved by the University of Arizona Institutional Animal Care and Use Committee and appropriate efforts were made to reduce animal suffering.

Nucleic acid isolation and quantitative polymerase chain reaction. RNA was extracted from frozen renal cortex in TRIzol isolated using a phenol-based centrifugation method (Life Technologies, Grand Island, NY). cDNA was reversed transcribed using the iScript Advanced cDNA Synthesis Kit (BioRad, Hercules, CA) and was added to a real-time SYBR green quantitative polymerase chain reaction master mix (BioRad). Changes in gene expression were calculated based on the Δ-Δ threshold cycle method. Primers are reported in Table 3-1.

Mouse tail tips were lysed using DirectPCR Lysis reagent (Viagen). Genomic DNA was amplified using Promega 2X PCR Master Mix in accordance with manufacturer's protocols. Amplified DNA was separated on a 2.5% agarose gel and visualized by ethidium bromide fluorescence.

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

Table 3-1. Primer sequences.

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

Protein isolation and immunoblotting. Frozen renal cortex was suspended in protein lysis buffer (1% Triton X-100, 150 mM NaCl, and 10 mM Tris-HCl, pH 7.4; 1 mM EDTA; 1 mM EGTA; 2 mM sodium orthovanadate; 0.2 mM phenylmethylsulfonyl fluoride; 1 mM HEPES, pH 7.6) containing protease inhibitors and phosphatase inhibitors (Sigma-Aldrich, St. Louis, MO). Following sonication, protein was quantified using a bicinchoninic acid assay, subjected to SDS-PAGE, transferred onto nitrocellulose membranes, and incubated with primary and secondary antibodies. Membranes were detected using chemiluminescence and processed using ImageJ (NIH, Bethesda, MD) software. Antibodies are reported in Table 3-2.

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

Histopathology. Kidney sections approximately 5–6 microns from animals at 144 hours after I/R or sham surgery were stained with hematoxylin and eosin and PAS, and the degree of morphologic changes was determined by light microscopy in a blinded fashion. Loss of brush border and necrosis were chosen as indicators of morphological damage to the kidney. These measures were evaluated on a scale from 0 to 4, which ranged from not present (0), mild (1), moderate (2), severe (3), and very severe (4).

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

Table 3-2. Antibodies

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

CHAPTER 4

CONCLUSIONS AND FUTURE DIRECTIONS

Summary of Relevant Findings

AKI occurs commonly in hospitalized patients and carries a high morbidity and mortality with no treatments beyond renal replacement therapy. AKI is characterized by mitochondrial dysfunction, particularly in the RPTC. The induction of MB is a therapeutic target for AKI. Our group has shown that formoterol, a β_2AR agonist, can induce MB in vitro and in vivo, and stimulate recovery of mitochondrial and renal function following AKI in mice.

Our in vitro study using rabbit RPTC elucidated the signaling pathway by which formoterol induces MB. The results showed that formoterol induces MB through a Gβγ-Akt-eNOS-cGMP-dependent pathway, while clenbuterol fails to activate this pathway. This study was the first to associate Gβγ- and cGMP-dependent signaling with β_2AR-induced MB.

Understanding differences in ligand bias enable more targeted application of pharmacotherapy to various disease states. Having identified differences in signaling between formoterol and clenbuterol, we hypothesized that these ligands interacted with the β_2AR at different residues. To understand the structural basis of these differences in signaling, formoterol and clenbuterol were docked to the orthosteric binding site of both inactive (3NYA, 3NY8, 5D5B) and active (4DLE, 4LDL, 4LDO) crystal structures of the β_2AR. For each pose, receptor-ligand interactions were tabulated at each structural feature. Each interaction was assigned a value of +1 for formoterol and -1 for clenbuterol, and interactions were summed for all six crystal structures. The methoxyphenyl group of formoterol showed a greater likelihood of aromatic interactions with W109 and V114 and contact interactions with F193. Interestingly, the formamide and hydroxyl groups of formoterol exhibited a greater interaction frequency with the backbone of C191, but the amino group of clenbuterol was more likely to engage in strong interactions at that site. Similarly, the secondary amino group of both formoterol and clenbuterol interacted with D113, but formoterol was more likely to do so with strong hydrogen bond or ionic interactions. Overall, the longer and larger functional groups of formoterol allowed it to stretch

across the binding pocket of the β_2AR receptor to simultaneously interact with TM3, TM5, and ECL2 in a manner distinct from clenbuterol and may drive the differences in signaling between formoterol and clenbuterol. These interaction profiles provide key structural data for the development of β_2AR agonists that selectively activate specific signaling programs.

AKI is the transient loss of renal function following an insult, such as nephrotoxicant exposure, sepsis, or ischemia. Numerous cell types play a role in the pathogenesis of AKI, including immune, endothelial, and RPTC. Our laboratory has demonstrated that the β_2AR agonist formoterol accelerates the recovery of renal function in mice following ischemia-reperfusion injury (IRI) with associated rescue of mitochondrial proteins. However, the cell type or types responsible for this recovery remain unknown.

To assess the role of RPTC in formoterol-induced recovery of renal function, we generated a proximal tubule-specific knockout of the β_2AR (γGT-Cre:ADRB2$^{Flox/Flox}$). These mice were subjected to renal IRI, followed by once daily dosing with formoterol beginning at 24 h and euthanasia at 144 h. Compared to wild-type controls (ADRB2$^{Flox/Flox}$), the γGT-Cre:ADRB2$^{Flox/Flox}$ mice had lower renal cortical mRNA expression of the β_2AR. Following IRI, vehicle-treated ADRB2$^{Flox/Flox}$ and γGT-Cre:ADRB2$^{Flox/Flox}$ mice exhibited a modest but incomplete recovery of renal function as measured by serum creatinine. Treatment with formoterol restored renal function in ADRB2$^{Flox/Flox}$ but not γGT-Cre:ADRB2$^{Flox/Flox}$ mice. Similarly, formoterol decreased renal injury as measured by KIM-1 and tubular necrosis in ADRB2$^{Flox/Flox}$ but not γGT-Cre:ADRB2$^{Flox/Flox}$ mice. Interestingly, treatment with formoterol restored brush border in both genotypes, albeit to a lesser extent in γGT-Cre:ADRB2$^{Flox/Flox}$ mice. Together, these data demonstrate that formoterol acts through RPTC β_2AR to exert its effects on renal function in AKI.

Many acute organ injuries and chronic degenerative diseases, including AKI, are associated with persistent disruptions in mitochondrial homeostasis. The induction of MB is therefore a promising therapeutic target for such diseases, particularly AKI. Our laboratory has demonstrated that treatment with the β_2AR agonist formoterol accelerates the recovery of renal function following AKI with associated rescue of mitochondrial protein expression. However, true MB (i.e., the formation of new mitochondria) has not been demonstrated. Mice lacking the β_2AR in proximal tubule cells (γGT-Cre:ADRB2$^{Flox/Flox}$) and wild-type controls (ADRB2$^{Flox/Flox}$) were subjected to renal IRI, followed by once daily treatment with formoterol beginning 24 h and euthanasia at 144 h. At 144 h, ADRB2$^{Flox/Flox}$ mice treated with formoterol exhibited recovery of mitochondrial DNA copy number (mtDNA) and the electron transport chain proteins NDUFS1 and COX1. However, γGT-Cre:ADRB2$^{Flox/Flox}$ mice treated with formoterol did not demonstrate recovery of mtDNA, NDUFS1, or COX1. Similarly, ADRB2$^{Flox/Flox}$ mice treated with formoterol demonstrated recovery of the mitochondrial fission and fusion proteins Drp1 and Mfn2, while expression of these markers was unaffected by formoterol treatment in γGT-Cre:ADRB2$^{Flox/Flox}$ mice. To determine whether the recovery of mtDNA and mitochondrial protein expression was due to increased MB, electron microscopy was used to quantify mitochondrial number and morphology. In ADRB2$^{Flox/Flox}$ mice but not γGT-Cre:ADRB2$^{Flox/Flox}$ mice, formoterol treatment increased both mitochondrial number and the total mitochondrial area following IR relative to vehicle-treated mice. In both genotypes, mice subjected to IRI regardless of treatment had increased mitochondrial roundness, consistent with injury. There was no difference in mitochondrial form factor among the groups, despite the changes in Drp1 and Mfn2 protein expression. Together, these data show for the first time that formoterol acts on RPTC β_2AR to induce MB to accelerate recovery of renal function following AKI.

These studies demonstrated that formoterol activates a G$\beta\gamma$-Akt-eNOS-cGMP signaling pathway to induce MB in RPTC. This pathway was not activated by all β_2AR agonists, and

activation of this pathway correlated with specific receptor-ligand interaction profiles. We demonstrated that formoterol acts on RPTC β_2AR to accelerate the recovery of renal function and mitochondrial protein expression. Finally, using electron microscopy, we demonstrated that formoterol increases mitochondrial number and density in RPTC. These studies demonstrate that specific GPCR-ligand interactions can drive distinct signaling pathways to affect mitochondrial homeostasis and organ function.

Future Directions

We have identified that formoterol, but not clenbuterol, activates a Gβγ-Akt-eNOS-cGMP pathway to induce MB. Furthermore, we found distinct receptor-ligand interaction profiles for β_2AR agonists that induce and do not induce MB. These data are suggestive of biased agonism through the β_2AR. The key determinant of ligand bias is reversal of efficacy.[633] In primary RPTC, both formoterol and clenbuterol increased cAMP accumulation, but clenbuterol was less efficacious than formoterol (Figure 4-1A). Treatment with formoterol, but not clenbuterol, also activated Akt in a concentration-dependent manner (Figure 4-1B), and neither agonist affected ERK1/2 phosphorylation (Figure 4-1C). These data fail to prove bias between the two ligands, as the increased efficacy of formoterol may reach a threshold to activate Akt phosphorylation. Furthermore, previous studies in other cell lines identified clenbuterol as a partial agonist for cAMP. Based on these data, further work is required to elucidate the potential impact of biased agonism on β_2AR-mediated MB.

While primary RPTC are a useful tool for the identification of inducers of MB and for confirmation of signaling pathways,[634] their recalcitrance to genetic manipulation (and therefore reliance on pharmacologic inhibitors) makes them a lesser model for elucidation of ligand bias. As such, cell lines must be identified that: recapitulate the effects of our panel of β_2AR agonists on MB; and demonstrate similar signaling as observed in RPTC following formoterol stimulation.

Once a model is developed, the efficacy of β_2AR agonists for various signaling pathways (e.g., cAMP/PKA, NO/cGMP, Ca^{+2}-dependent signaling) can be identified. Based on efficacy profiles, signaling pathways that are activated by inducers of MB can be identified. Furthermore, agonists can be clustered based on their efficacy profiles, and these clusters can be compared to pharmacophore models and docking experiments to identify structural determinants of these signaling pathways. Using immortalized cell lines also allows for elucidation of the effects of formoterol and other β_2AR agonists on G protein, arrestin, and kinase recruitment to the β_2AR.

Pre-treatment with the GRK2 inhibitor Cmpd101 prevented formoterol-induced increases in FCCP-OCR without affecting Akt phosphorylation (Figure 4-2), suggesting that formoterol activates GRK2 in a pathway distinct or downstream from Akt. Understanding whether this is due to a direct interaction between GRK2 and the receptor or through some other pathway would provide important mechanistic insights to GPCR-mediated MB.

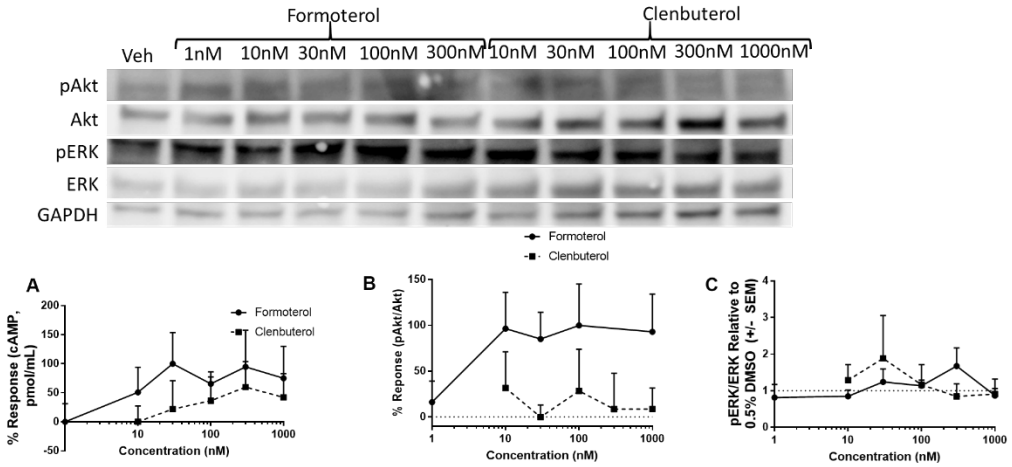


Figure 4-1. The effects of formoterol on cAMP accumulation and Akt and ERK1/2 phosphorylation. RPTC were treated with formoterol or clenbuterol for 30 min. Cyclic nucleotides and protein were extracted. cAMP accumulation was measured by ELISA (A). Immunoblot analysis was used to assess the phosphorylation of Akt (B) and ERK1/2 (C). N=3-6. No statistical significance between drugs was determined by Two-Way ANOVA.

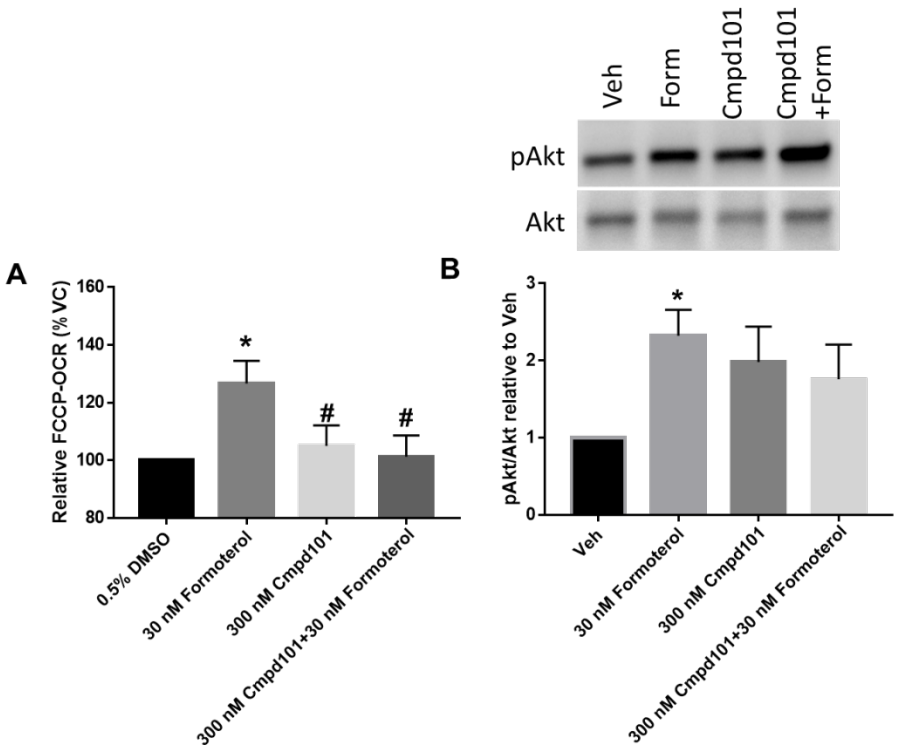


Figure 4-2. The effects of GRK2 inhibition on formoterol-induced MB and Akt phosphorylation. A. RPTC were pre-treated with 300 nM Cmpd101 (a GRK2/3 inhibitor) for 30 min, followed by treatment with formoterol for 24 h. FCCP-OCR was measured using the Seahorse XF96 analyzer. B. RPTC were pre-treated with 300 nM Cmpd101 for 30 m, followed by treatment with formoterol for 30 min. Akt phosphorylation was assessed by immunoblot. Mean $\pm$ SEM, N=4-6. *-$p<0.05$ vs. Vehicle, #- $p<0.05$ vs. 30 nM Formoterol, One-Way ANOVA with Sidak's multiple comparisons test.

Formoterol accelerates the recovery of renal function in mice subjected to IR-induced AKI. However, there are numerous causes of AKI, and no single mouse model fully recapitulates human disease. The disconnect between mouse models and human disease is one of the obstacles to the development of effective therapeutics for AKI. As such, the effects of formoterol in other models of AKI should be explored. Activation of the β_2AR by terbutaline or overexpression is renoprotective in mouse models of septic AKI,[578-580,635-637] however, β_2AR activation reduces creatinine clearance in healthy rats.[584] Because formoterol activates G$\beta\gamma$-dependent signaling distinct from other β_2AR agonists, the detrimental effects of β_2AR signaling observed in the rat study may be ameliorated by treatment with formoterol. Additionally, preliminary data in proximal tubule cell lines demonstrated that administering formoterol after LPS decreases TNF-α mRNA expression at 24 h (Figure 4-3). These data indicate that formoterol may have utility in models of non-ischemic AKI, thereby expanding its future clinical utility.

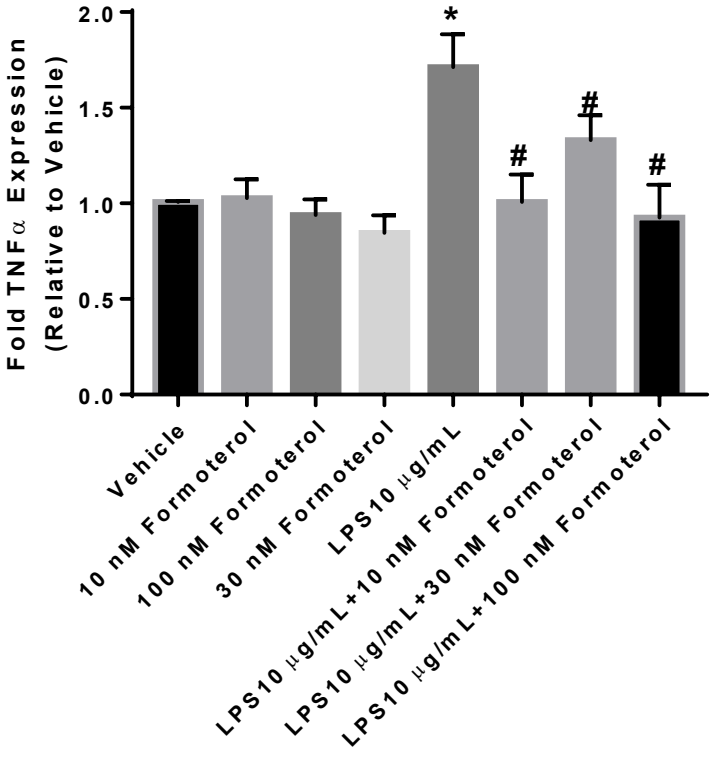


Figure 4-3. Formoterol decreases TNFα expression following LPS administration in TKPTS cells. The proximal tubule TKPTS cell line was treated with LPS (10 μg/mL) for 1 h, followed by treatment with 10, 30, or 100 nM formoterol. After 24 h, mRNA was extracted, and TNFα expression was measured by RT-qPCR. Mean±SEM, N=5-6. *-$p<0.05$ vs. Vehicle, #-$p<0.05$ vs LPS 10 μg/mL, One-Way ANOVA.

Formoterol is used clinically for the treatment of asthma and COPD, but its use is limited by a black-box warning for severe asthma exacerbations. Because airway smooth muscle expresses high levels of β_2AR, current inhaled formulations are designed to minimize systemic distribution of formoterol. However, for AKI, an intravenous or oral formulation would be required to achieve sufficient drug concentrations in RPTC. Additionally, since its patent has expired, formoterol is not an attractive lead compound for drug companies. Two studies would help overcome this barrier to clinical translation. One is employing electronic health record databases to identify potential correlations between formoterol use and renal dysfunction. Based on our preclinical results, we expect that formoterol use is negatively correlated with the development of AKI. However, identifying proper patient controls and obtaining a sufficient patient population to achieve statistical power may prove challenging.

Another study to increase the likelihood of successful application of formoterol as treatment for AKI is the development of a targeted formulation of formoterol. Recent work has identified nanoparticle formulations that selectively target RPTC;[638] however, new formulations may be necessary to target plasma membrane receptors, as opposed to intracellular targets. A new formulation could be patented by drug companies, facilitating the funding of potentially costly clinical trials.

While most patients recover from their initial bout of AKI, they are sensitized to future renal and cardiovascular dysfunction, and some patients progress from AKI into CKD. Formoterol accelerates the recovery of renal function in the acute recovery phase of AKI. The effects of formoterol on the AKI-CKD transition, cardiorenal syndrome, and CKD itself are not known. Identifying the therapeutic effects of formoterol in these disease processes would allow for more effective identification of patients who would benefit from formoterol administration.

We generated a mouse with a RPTC-specific deletion of the β_2AR and observed that RPTC β_2AR is necessary for the effects of formoterol on renal function and MB following AKI.

Healthy mice lacking RPTC β_2AR had normal renal function as measured by SCr; however, we observed an increase in NDUFS1 protein expression and increased mitochondrial length. Additionally, these mice had fewer mitochondria and a lower mitochondrial density. Given the oxidative state of RPTC, it is possible that mice lacking RPTC β_2AR have a subclinical disruption in mitochondrial homeostasis that disrupts solute transport and sensitizes the kidney to injury. The β_2AR has been implicated in renal sodium transport and Na^+/K^+-ATPase expression.[639,640] Urinalysis could be performed on $ADRB2^{Flox/Flox}$ and γGT-Cre:$ADRB2^{Flox/Flox}$ mice to identify changes in urinary electrolyte concentrations.

Additional experiments could be performed on male and female mice of varying ages to identify any sex differences or age-dependent differences in renal function caused by lack of the β_2AR. These experiments would provide an important link between mitochondrial homeostasis and basic renal physiology, and these data will inform potential effects of β_2AR polymorphisms on renal function.

Endothelial and immune cells play important roles in both the injury and recovery phases of AKI.[139,624] Because the β_2AR is ubiquitously expressed, it is reasonable to infer that formoterol exerts an effect on endothelial and immune cell function in AKI and in other diseases. Experiments could identify levels of different circulating cytokines at different times after AKI and how formoterol affects these cytokines. Because cytokines are secreted by and affect the infiltration of different immune cell types, these data will allow for a more focused examination of infiltrating immune cells following AKI.

Administration of formoterol following AKI increases the protein expression of electron transport chain proteins as well as proteins associated with mitochondrial fission and fusion. However, we found that formoterol did not affect the mRNA expression of PGC-1α, COX1, NDUFS1, or ND1 following IR (Figure 4-4). Because tissue was taken after multiple doses of formoterol, these findings may be due to a negative feedback pathway following formoterol-

induced MB that prevent excessive mitochondrial content. Similarly, these findings could be due to desensitization of the β_2AR following stimulation by formoterol.

Another potential explanation for these findings is the effect of formoterol on mitophagy, or the clearance of damaged mitochondria. Controlled mitophagy is generally renoprotective,[212] but excessive or uncontrolled mitophagy leads to a lethal energy imbalance and subsequent cell death.[641] The findings of recovered mitochondrial protein but not mRNA expression may suggest that formoterol can inhibit mitophagy. Mitophagy is inhibited by mammalian target of rapamycin (mTOR), which is a known downstream target of Akt. Because formoterol increases Akt phosphorylation, formoterol may play a role in the regulation of mitophagy.

To assess the role of mitophagy on the effects of formoterol, protein expression of markers of autophagy (e.g., LC3BII, p62, and p-mTOR) could be measured. To confirm that changes in these proteins are due to mitophagy rather than general autophagy, the MitoTimer mouse could also be used. This mouse has a mitochondrial fluorescent protein that shifts from green to red fluorescence following oxidative stress,[642] allowing for the visualization of old or damaged mitochondria. In this system, red punctate mitochondria are generally considered to be targeted for lysosomal degradation. These mice could therefore be used to quickly visualize mitochondria in multiple organs to determine the effects of formoterol or other inducers of MB on mitochondrial content, morphology, and degradation.

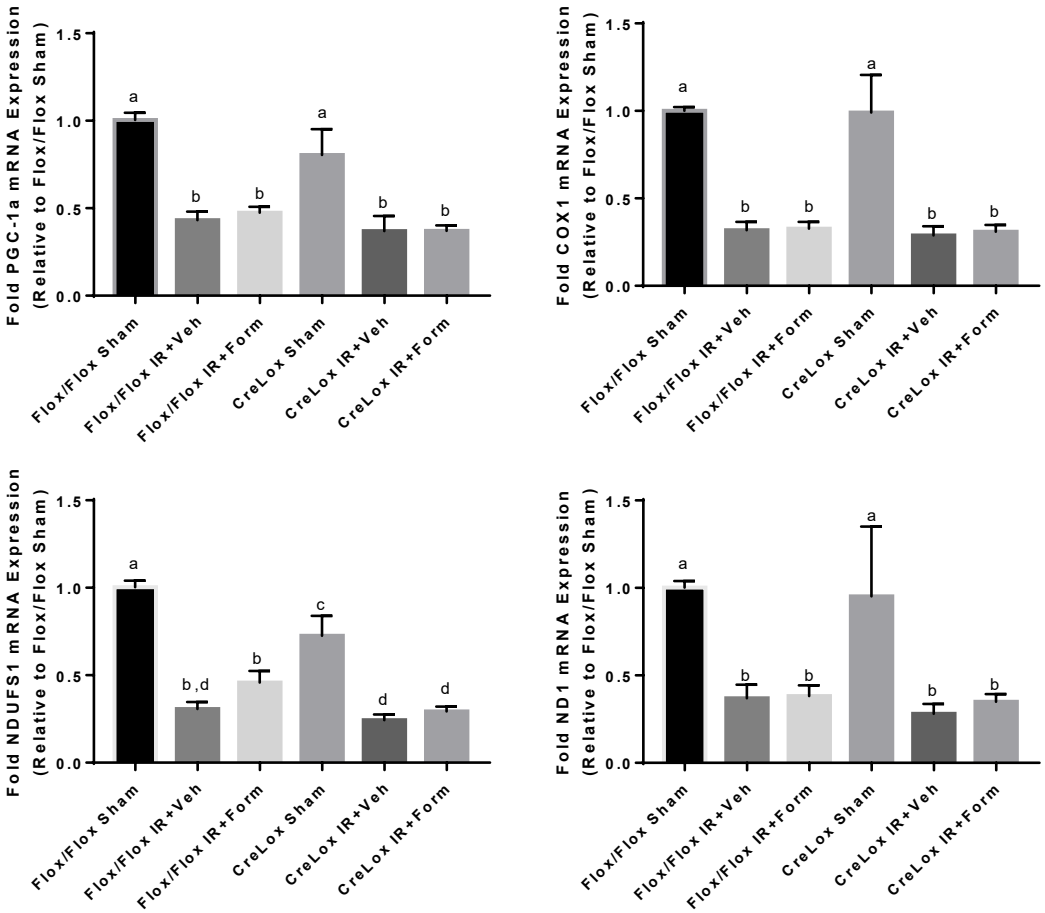


Figure 4-4. Formoterol does not increase mRNA expression of markers of MB following AKI. ADRB2$^{Flox/Flox}$ (Flox/Flox) and γGT-Cre:ADRB2$^{Flox/Flox}$ (CreLox) mice were subjected to renal IR followed by treatment with formoterol. mRNA expression of PGC-1α, COX1, NDUFS1, and ND1 was measured by RT-qPCR in renal cortex 144 h post-injury. Mean ± SEM. N=3-9. Different subscripts indicate $p<0.05$, Two-Way ANOVA followed by Fisher's LSD test.

We identified the Gβγ-Akt-eNOS-cGMP pathway as necessary for formoterol-induced MB in vitro. Other work in our lab has identified this pathway as important for $5HT_{1F}$ receptor-mediated MB in cultured RPTC.[541] The importance of this pathway in vivo remains unclear. Preliminary data demonstrated that formoterol increases Akt phosphorylation in mouse renal cortex 30 min after administration and that this phosphorylation is blocked by pretreatment with gallein (Figure 4-5A, C); furthermore, mice have elevated cGMP levels at 30 and 60 min following formoterol administration (Figure 4-5B). At 24 h following formoterol administration, renal cortical ATPSβ increased, and this increase was also blocked by pretreatment with gallein (Figure 4-5D). To examine the effects of formoterol in AKI, mice were subjected to IRI, followed by treatment with formoterol or diluent 24 h after injury. At 30 min after drug administration, Akt phosphorylation was elevated regardless of treatment; however, mice treated with formoterol exhibited decreased ERK1/2 phosphorylation (Figure 4-6A). In mice treated with formoterol, there was decreased renal injury and recovery of renal function and mitochondrial protein expression 24 h following drug administration (Figure 4-6B-D). Further work is necessary to determine the signaling pathway(s) activated by formoterol in the injured kidney and the role of this signaling in recovery of renal function.

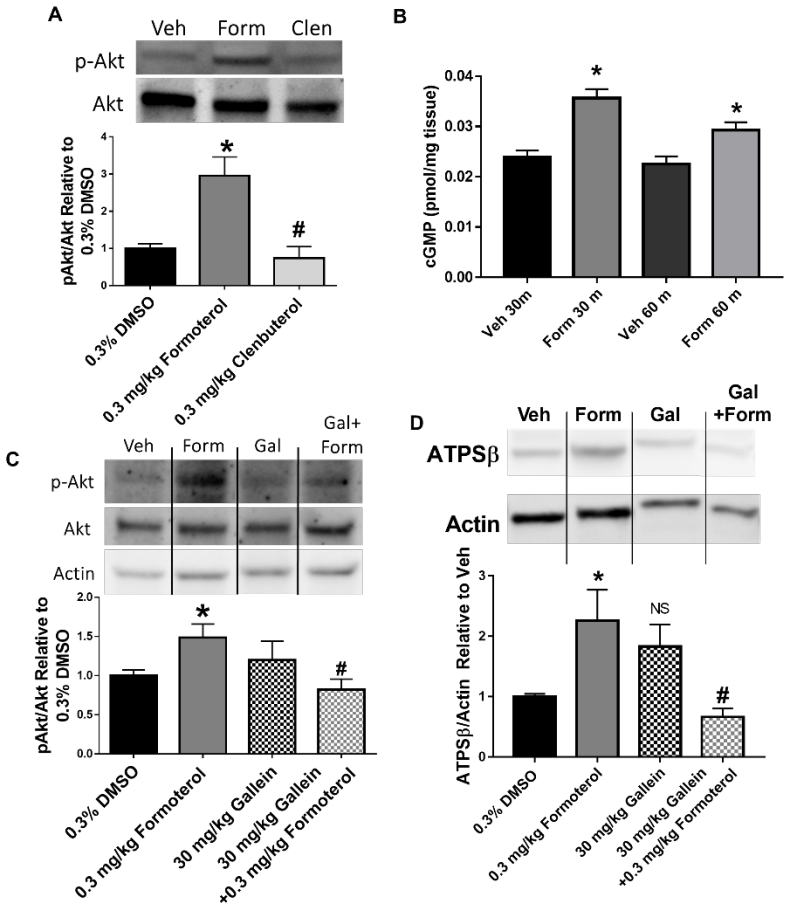


Figure 4-5. Formoterol activates Akt in a Gβγ-dependent manner to induce MB in vivo. A. Male C57Bl/6 mice were treated with 0.3 mg/kg formoterol or 0.3 mg/kg clenbuterol for 30 min. Akt phosphorylation at S473 was measured in renal cortex. B. Male C57Bl/6 mice were treated for 30 min or 1 h with 0.3 mg/kg formoterol. Accumulation of cGMP in the renal cortex was measured by ELISA. C. Male C57Bl/6 mice were pre-treated with 30 mg/kg gallein for 1 h followed by treatment with 0.3 mg/kg formoterol for 30 min. Akt phosphorylation was measured at S473 in renal cortex. D. Male C57Bl/6 mice were pre-treated with 30 mg/kg gallein for 1 h followed by treatment with 0.3 mg/kg formoterol for 24 h. ATPSβ levels were measured in the renal cortex. Mean ± SEM. N=4-6 *- $p<0.05$ vs. 0.3% DMSO, #- $p<0.05$ vs 0.3 mg/kg Formoterol.

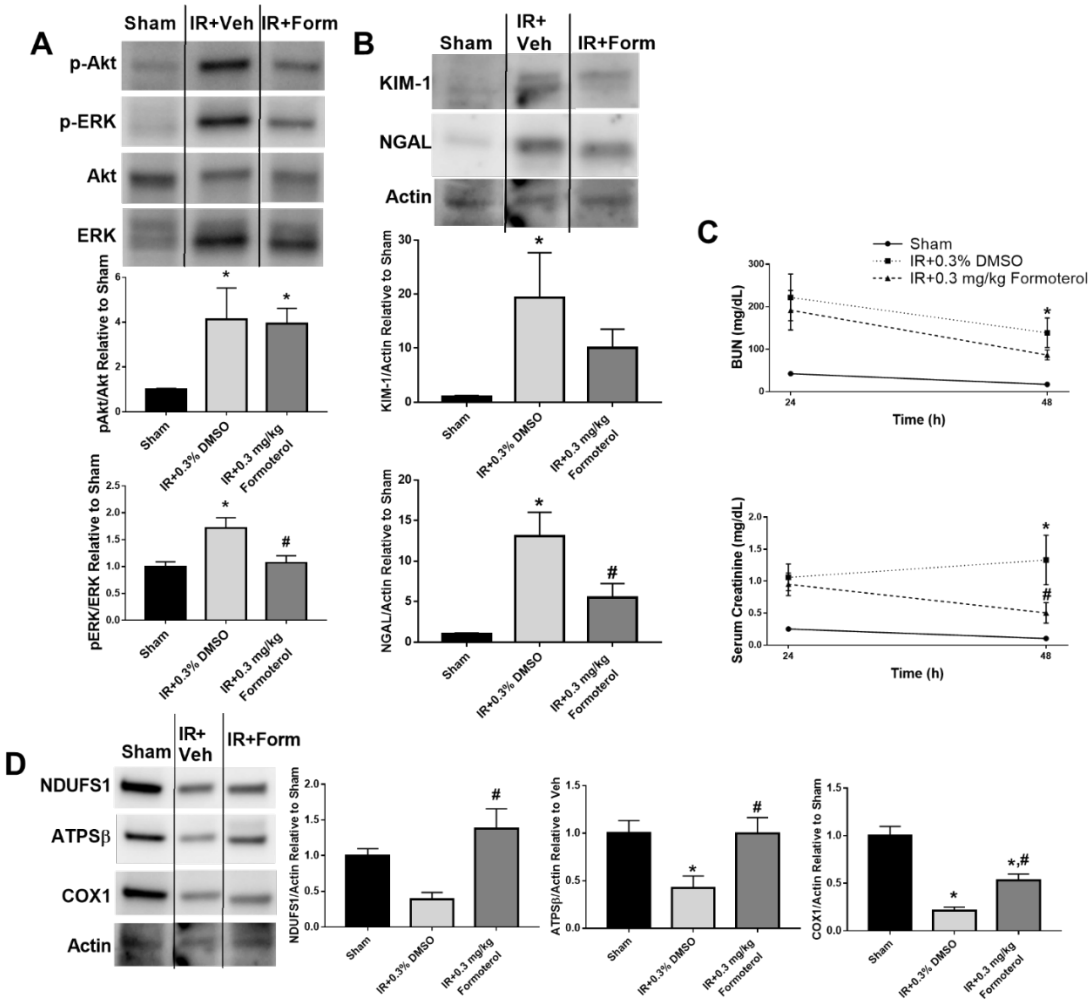


Figure 4-6. Formoterol modulates ERK1/2 phosphorylation, renal function, and MB early after AKI. Male C57Bl/6 mice were subjected to 18.5 min of warm ischemia followed by reperfusion. 24 h after injury, 0.3 mg/kg formoterol was administered. A. Mice were euthanized 30 min after formoterol administration, and renal cortical Akt and ERK1/2 phosphorylation were measured by immunoblot. B. Mice were euthanized 24 h after formoterol administration. Renal cortical expression of the injury markers KIM-1 and NGAL was assessed by immunoblot analysis. C. Serum was obtained 24 h after AKI and 24 h after formoterol administration. BUN and SCr were measured. D. Mice were euthanized 24 h after formoterol administration, and renal cortical mitochondrial protein expression was measured by immunoblot analysis. Mean $\pm$ SEM. N=4-8 *- $p<0.05$ vs. Sham, #- $p<0.05$ vs IR+0.3%DMSO, One-Way ANOVA with Fisher's LSD test.

Numerous drugs are known to induce MB, and we have identified several compounds that induce MB to accelerate the recovery of renal function following AKI. Among these compounds are the SIRT1 activator SRT1720,[230] the MEK/ERK inhibitor trametinib,[632] the phosphodiesterase inhibitor sildenafil, the $5HT_{1F}$ receptor agonist LY344864, and the β_2AR agonist formoterol. Both formoterol and LY344864 are known to activate Gβγ-Akt-eNOS-cGMP-dependent signaling to induce MB in vitro;[541] however, while formoterol increases cAMP through $G\alpha_s$-dependent signaling, LY344864 activates the inhibitory $G\alpha_i$ and also inhibits ERK1/2 phosphorylation. All of these compounds activate signaling pathways that converge upon PGC-1α to induce MB. However, it is unlikely that these drugs only upregulate genes associated with MB. It is therefore important to determine other potentially beneficial or deleterious pathways activated by these compounds. For example, mice could be subjected to renal IR (or other organ injury) and treated with different inducers of MB. Tissue from these mice would then be subjected to transcriptomic analysis to identify transcriptional networks that are affected by these compounds. Using a panel that includes GPCR ligands as well as more targeted drugs, the data may be used to determine the roles of specific components of signaling pathways on gene expression. Due to the heterogeneous population of AKI patients, these data will help identify more specific patient populations to facilitate future clinical studies.

CHAPTER 5

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