REGULATION OF MITOCHONDRIAL DYNAMICS IN THE HYPERGLYCEMIC RENAL PROXIMAL TUBULE BY THE β_2-ADRENERGIC RECEPTOR AGONIST FORMOTEROL

by

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A Dissertation Submitted to the Faculty of the

DEPARTMENT OF PHARMACOLOGY AND TOXICOLOGY

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

In the Graduate College

THE UNIVERSITY OF ARIZONA

2022

Acknowledgments

I first and foremost want to thank my mentor, Dr. Rick Schnellmann for his guidance and mentorship throughout my dissertation. I have been lucky enough to work in Rick's lab over the last five years and the amount that I have learned from him is overwhelming. He has continuously pushed me to work hard and although at times it was difficult, his mentorship has allowed me to become a better researcher and I am truly thankful for these experiences. I also thank my committee members (Drs. Frank Brosius, Richard Vaillancourt, Jim Galligan and Heddwen Brooks) as they have provided me with valuable scientific feedback that has allowed me to grow as an independent researcher. I would like to thank the members of the Schnellmann lab, particularly: Drs. Natalie Scholpa and Jaroslav Janda, Kiara Bachtle, Paul Victor, Ingrid Peterson and Austin Thompson. I specifically want to thank Kevin Hurtado for his friendship and presence over the last four years. Lab would have been a different and less entertaining place to be without him around. I want to thank my best friend Dr. Epiphani Simmons for her friendship and emotional support. Importantly, I want to thank Marsh and Anjunadeep, I can't imagine my life without them. Finally, I would like to thank my family for their support.

Dedication

This dissertation is dedicated to my one and only Koba Fish and Ryan Bosler, MD.

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Abbreviations:

5-Hydroxytryptamine-1F receptor (5-HT1FR)

β2-adrenergic receptor (β2-AR)

β-arrestin 1 and 2 (βarr1/2)

Acute kidney injury (AKI)

AMP-activated protein kinase (AMPK)

Angiotensin I (Ang I)

Angiotensin II (Ang II)

Angiotensin converting enzyme (ACE)

Angiotensin receptor blocker (ARB)

Angiotensin type 1 receptor (AT1R)

Angiotensin type 2 receptor (AT2R)

Antimycin A (AA)

Ca^{2+}/calmodulin-dependent kinase II (CaMKII)

Carbonyl cyanide p-trifluoro-methoxyphenyl hydrazone-oxygen consumption rates (FCCP-OCR)

Chronic kidney disease (CKD)

Canagliflozin and Renal Events in Diabetes with Established Nephropathy Clinical Evaluation (CREDENCE)

Dapagliflozin and Prevention of Adverse Outcomes in Chronic Kidney Disease (DAPA-CKD)

Diabetic kidney disease (DKD)

Dynamin-like protein 1 (Drp1)

Empagliflozin Outcome Trial in Patients with Chronic Heart Failure with Reduced Ejection Fraction (EMPEROR-Reduced)

Electron transport chain (ETC)

Endothelial nitric oxide synthase (eNOS)

End-stage renal disease (ESRD)

Epidermal growth factor (EGF)

Glomerular filtration rate (GFR)

Insulin-like growth factor 1 (IGF1)

Ischemia/reperfusion (IR)

Kidney Disease: Global Outcomes (KDIGO)

Mitochondrial biogenesis (MB)

Mitochondrial membrane permeability (mtMP)

Mitofusin 1 (Mfn1)

Mitofusin 2 (Mfn2)

NADPH oxidase 2 (Nox2)

Optic atrophy 1 (Opa1)

Oxidative phosphorylation (OxPhos)

Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α)

Phorbol myristate acetate (PMA)

Platelet derived growth factor (PDGF)

PTEN-induced kinase 1 (PINK1)

Protein kinase A (PKA)

Protein kinase B (PKB)

Reactive oxygen species (ROS)

Receptor interacting protein 140 (RIP140)

Renal proximal tubule cell (RPTC)

Renin angiotensin system (RAS)

Rho-associated protein kinase (ROCK1)

Soluble gualynate cyclase (sGC)

Sodium glucose co-transporter-1 (SGLT1)

Sodium glucose co-transporter-2 (SGLT2)

Sirtuin 1 (SIRT1)

Streptozotocin (STZ)

Transforming growth factor beta-1 (TGFβ1)

Tumor necrosis factor alpha (TNFα)

Type 2 diabetes mellitus (T2DM)

Type 2 diabetic nephropathy (T2DN)

Abstract

Diabetic kidney disease (DKD) is the leading cause of end-stage renal disease (ESRD) in the United States and many other countries. DKD occurs through a variety of pathogenic processes that are in part driven by hyperglycemia and glomerular hypertension, leading to gradual loss of kidney function and eventually progressing to ESRD. In type 2 diabetes, chronic hyperglycemia and glomerular hyperfiltration leads to glomerular and proximal tubular dysfunction. Simultaneously, mitochondrial dysfunction occurs in the early stages of hyperglycemia and has been identified as a key event in the development of DKD. Clinical management for DKD relies primarily on blood pressure and glycemic control through the use of numerous therapeutics that slow disease progression. Because mitochondrial function is key for renal health over time, therapeutics that improve mitochondrial function could be of value in different renal diseases. Increasing evidence supports the idea that targeting aspects of mitochondrial dysfunction, such as mitochondrial biogenesis and dynamics, restores mitochondrial function and improves renal function in DKD. We will review mitochondrial function in DKD and the effects of current and experimental therapeutics on mitochondrial biogenesis and homeostasis in DKD over time.

Chapter 1:
Introduction

1.1 Overview

Diabetic kidney disease (DKD) is the leading cause of end stage renal disease (ESRD) in the United States with insufficient therapies for preventing its progression. In DKD, hyperglycemia affects mitochondrial dynamics, leading to mitochondrial dysfunction. Mitochondria constantly undergo biogenesis, fission and fusion to change their size and shape in response to metabolic and environmental signaling. Maintaining a proper balance between biogenesis, fission and fusion is essential for proper mitochondrial homeostasis and defective mitochondrial biogenesis and dynamics have been associated with DKD (1-3).

While previous studies have focused on DKD as a glomerulopathy, there is significant evidence that the proximal tubule plays an important role in the pathogenesis of DKD (4-7). Increases in blood glucose directly affects proximal tubular reabsorption and function, leading to poorer prognosis, particularly in early DKD. While therapies for DKD primarily rely on targeting hypertension and hyperglycemia, these drugs only slow disease progression. Thus, there is a need for the identification of new therapeutics to treat DKD. Mounting evidence suggests that improving mitochondrial function in the early stages of hyperglycemia leads to improved renal function, supporting the use of mitochondrial-targeted therapies for DKD.

1.2 Pathophysiology of Diabetic Kidney Disease

1.2.1 Mitochondrial Homeostasis in the Kidney

Mitochondria are highly dynamics organelles that are constantly adapting to different cellular and environmental cues. The kidney, being the second most energetically demanding organ in the body, requires constant ATP to transport solutes along the nephron (8, 9). Mitochondria are densely located in the renal proximal tubule, which are required to reabsorb glucose, NaCl and ions to maintain proper physiological homeostasis. Therefore, maintaining mitochondrial function and homeostasis are essential for renal function.

One of the key factors in regulating mitochondrial homeostasis is a process known as mitochondrial dynamics. Mitochondria continuously undergo the processes of fission and fusion to get rid of old and damaged mitochondria, and to promote mitochondrial fitness, respectively. Mitochondrial fission is primarily mediated by the GTPase dynamin-like protein 1 (Drp1). During fission, Drp1 resides in the cytosol and translocates to the outer mitochondrial membrane upon activation where it utilizes GTP hydrolysis to form ring-like oligomers to scission the membrane (10,155, 169). During its translocation, Drp1 is recruited to the mitochondrial membrane by several Drp1 receptors, including Fis1, MFF and MiD49/MiD51. Once bound to its receptors, Drp1 is able to carry out mitochondrial fission (1, 11, 12, 168).

Drp1 activity is regulated by a variety of post-translational modifications including sumoylation, ubiquitination, and S-nitrosylation, with the primary and most well-studied regulatory event being phosphorylation (13-15). Phosphorylation of Drp1 can lead to

multiple functional outcomes: activation or inhibition of its activity, and this occurrence is both context and stimulus dependent. Phosphorylation of Drp1 at Ser637 by protein kinase A (PKA) has been shown to inhibit Drp1 activation and subsequent translocation to the mitochondria, preventing its ability to initiate mitochondrial fission (16). Contrary to this finding, studies have found that in hyperglycemic conditions, Drp1 phosphorylation at Ser637 (corresponding to S600 in mice) by the upstream kinase Rho-associated protein kinase 1 (ROCK1) activates Drp1, thereby promoting its mitochondrial translocation and resulting in increased fission (2, 3, 17). These differential findings highlight the importance of both the context and stimulus specificity of Drp1 phosphorylation. Drp1 activity is also regulated by phosphorylation at an additional site, Ser616. Phosphorylation of Ser616 is regulated by a variety of upstream kinases, including Ca^{2+}/calmodulin-dependent kinase II (CaMKII) and PTEN-induced kinase 1 (PINK1) (18). Increased fission events as a result of Drp1 dysregulation have shown to be a key occurrence in the progression of DKD in mice (Fig. 1), and blocking excessive Drp1 Ser637 phosphorylation promotes mitochondrial function and prevents disease progression (2, 3, 17).

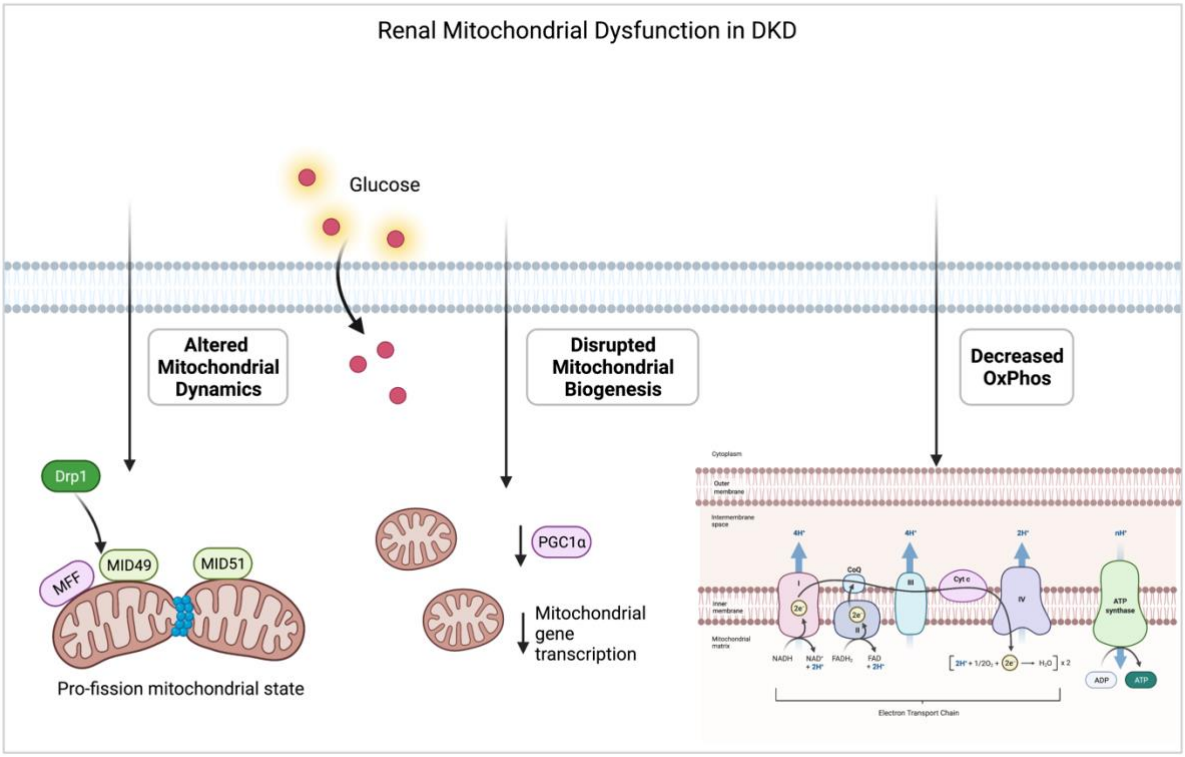


Figure 1.1 Renal mitochondrial dysfunction in response to hyperglycemia. Increasing concentrations of blood glucose leads to altered mitochondrial dynamics and increased Dynamin-like protein 1 (Drp1) activation, promoting a pro-fission mitochondrial state. Disrupted mitochondrial biogenesis leads to decreased expression and activity of peroxisome proliferator-activated receptor gamma coactivator (PGC1α) and transcription of genes encoding mitochondrial genes. Elevated glucose disrupts electron transport chain activity, leading to decreased oxidative phosphorylation.

The opposing player in mitochondrial dynamics is mitochondrial fusion, which is primarily mediated by two dynamin-like GTPases mitofusin 1 and mitofusin 2 (Mfn1/Mfn2). In the event of mitochondrial fusion, Mfn1 and Mfn2, working in homo or heterodimers, become activated and promote fusion of the outer mitochondrial membrane (1, 167), which is also a GTP-dependent process. Fusion of the inner mitochondrial membrane is facilitated by optic atrophy 1 (Opa1), another dynamin-related GTPase (1). Mitochondrial fusion allows for elongation of mitochondria and can help maintain OxPhos (153). In addition, mitochondrial fusion also plays a role in maintaining mitochondrial energetics. A study by Mourier et al. showed that knockdown of Mfn2 leads to deficient coenzyme Q, a subunit of ETC complex III, further leading to altered ETC activity and decreased ATP production (154). Mitochondrial fusion has not been as extensively studied as Drp1 in the context of DKD and hyperglycemia, but a few studies have identified that expression of Mfn1 decreases in response to high glucose (19, 20), indicating mitochondrial fusion may also be a contributing factor leading to mitochondrial dysfunction in hyperglycemic conditions.

Since mitochondria are refined to meet the bioenergetic demands of the cell, they are not only constantly adapting to the cellular environment through continuous fission and fusion, but also through mitochondrial biogenesis (MB), the generation of new and functional mitochondria. MB is largely mediated by a set of interconnected transcription factors that encompass the peroxisome proliferator-activated receptor γ (PPARγ) coactivator-1 family, including PGC1α, PGC1β and PRC. Among this set of proteins, PGC1α is widely known as the master regulator of MB (8). PGC1α has been extensively studied in the kidney and its activation has been shown to have beneficial effects on renal

function. Under normal physiological conditions, PCG1α responds to environmental stimuli, and in turn be activated by a variety of signal transduction pathways including cAMP/PKA/CREB, eNOS/sCG/cGMP, 5-AMP-activated protein kinase (AMPK) or acetylation by sirtuin 1 (SIRT1) (21-23). Despite the multitude of potential upstream activators, PGC1α routinely is phosphorylated and translocates to the nucleus where it initiates transcription of genes responsible for MB and ATP production (24). Due to its key role in mitochondrial function and homeostasis in the kidney, PGC1α downregulation has been identified in the development and progression of DKD. Studies report that PGC1α is downregulated in the proximal tubules of streptozotocin (STZ)-induced rats, as well as in diabetic db/db mice and pharmacological activation of PGC1α has a beneficial effect on renal function (25).

Mitophagy, the selective removal of mitochondria by autophagy, is also an important component of mitochondrial homeostasis. Mitophagy is primarily regulated by PINK1/Parkin signaling (26, 27). Under normal physiological conditions, PINK1 resides in the cytosol and translocates to the mitochondria where it is tagged for degradation (28). Upon mitochondrial depolarization, PINK1-induced phosphorylation of mitochondrial proteins, such as Mfn1 and Mfn2, recruits the E3 ubiquitin ligase Parkin to the mitochondrial membrane where it targets mitochondrial for autophagosomal degradation (29, 30). It is currently understood that mitophagy plays a protective role in the kidney and its dysfunction contributes to renal cell dysfunction and disease progression. Several studies have found evidence for dysfunctional mitophagy in the kidneys of diabetic mice, specifically in the renal tubules (31, 32). These changes were accompanied by altered expression of the mitophagy proteins PINK1, Parkin, LC3II and Beclin1 (32). Evidence

also exists for dysfunctional mitophagy in podocytes of diabetic mice, where knockdown of Parkin inhibited mitophagy, increased the number of apoptotic podocytes and increased ROS (33). Compounds that induce mitophagy, such as resveratrol and berberine, have shown to be protective against DKD (34, 35). Interestingly, these compounds can also regulate mitochondrial function by inducing of PGC1α and inhibiting of mitochondrial fission, respectively (36, 37).

1.2.2 Effect of Hyperglycemia on Mitochondrial Homeostasis

Mitochondria play a critical role in maintaining renal function, as the kidney requires substantial amounts of oxygen to convert to energy to meet the metabolic demands of the organ. Oxygen is used by oxidative phosphorylation (OxPhos) in the mitochondria to convert fatty acids, glucose and amino acid substrates to ATP. During these metabolic processes, oxygen is consumed by the electron transport chain (ETC), acting as an electron acceptor in the molecular reactions carried out by ETC complexes I-IV. ETC complex V (ATP synthase) then uses this proton gradient generated by ETC complexes I-IV to produce ATP, which is subsequently exported from the mitochondria. The majority of ATP produced by OxPhos is used in the renal proximal tubule to reabsorb glucose, NaCl and ions from the glomerular filtrate (38, 39). Hyperglycemia causes ETC dysfunction. Increases in blood glucose lead to increased production of NADH and $FADH_2$ by the TCA cycle, which in turn leads to increased ETC activity (156). The increase in ETC activity further leads to an increase in ROS production and release from the ETC, which can induce mitochondrial DNA and protein damage (156).

Along with reabsorption, proximal tubule cells use ATP to produce glucose via gluconeogenesis (40). In hyperglycemic conditions, the normal gluconeogenic mode of metabolism is shifted so that both gluconeogenesis and glycolysis take place simultaneously (41, 42). As a result of this metabolic shift, proximal tubule cells are less efficient at producing and maintaining energy to support normal physiological functions.

In animal models of DKD, hyperglycemia leads to renal mitochondrial dysfunction in multiple cell types along the nephron (2, 3, 17, 19, 43). A study using diabetic rats with elevated blood glucose showed decreased mitochondrial membrane potential and increased levels of reactive oxygen species (ROS). These effects were accompanied by both tubular morphological changes and tubular functional impairment (43). In addition, a study by Ayanga et al. using diabetic db/db mice had dysfunctional mitochondria in podocytes. Mitochondrial dysfunction was characterized by increased Drp1 expression, increased mitochondrial fission and decreased mitochondrial respiration, further leading to decreased ATP production (2). The authors found that knockdown or pharmacological inhibition of Drp1 restored these altered mitochondrial processes, leading to improved mitochondrial fitness. Importantly, they show that by knocking out Drp1, there was a significant reduction in albuminuria and histopathological changes such as mesangial matrix expansion, improvement in podocyte foot process effacement and a significant reduction in glomerular basement membrane thickness. A more recent study demonstrated that mutation of Drp1 S600 (S637) in which the serine was mutated to an alanine, diabetic mice showed significantly improved albuminuria, attenuated mesangial matrix expansion and preserved podocyte structure (3), similar to what was observed in Drp1 knockout mice in the former study.

Recent studies from our laboratory show that in 13 week old diabetic db/db mice, representing a very early model of DKD, phosphorylation of Drp1 and ETC complex subunit expression were increased, and expression of Mfn1 as well as ATP production were decreased in renal cortical tissue (19). Similarly, when renal proximal tubule cells (RPTC) in culture were subjected to high glucose injury, the same pattern of mitochondrial changes were observed, indicating that mitochondrial dysfunction begins in the earliest phase of hyperglycemia before pathological changes have occurred. A study that evaluated metabolic flux in diabetic kidneys of 12 week vs 24 week diabetic mice reported that ETC complexes I, II and III expression were significantly decreased (42). These differences suggest that the early increase in ETC complex expression in our model may be a result of increased glucose metabolic flux, followed by mitochondrial damage. Interestingly, the authors also reported that TCA cycle metabolites and acylcarnitines were increased in 24 week diabetic mice compared to 12 week, suggesting that in opposition to mitochondrial ETC, mitochondrial metabolism progressively increased as the need for energy production increases.

1.2.3 Glomerular Hyperfiltration and Proximal Tubular Injury

The daily glomerular filtrate contains ~180 mg of glucose, which is reabsorbed by the proximal tubule to prevent loss into the urine (44). Glucose reabsorption along the proximal tubule is through the sodium-glucose cotransporter 2 (SGLT2) and sodium-glucose cotransporter 1 (SGLT1) and is proceeded by facilitated diffusion via GLUT2 on the basolateral membrane (45) (Fig. 2). SGLT2, the primary mediator of glucose reabsorption in the early segments, is mostly confined to the proximal tubule and has a

low affinity, yet high capacity for glucose uptake (46, 47). This capacity is reached at >12mmol/l (200mg/dl) in a hyperglycemic patient (48). The remaining glucose is taken up by the high affinity low capacity SGLT1 in the later segments. In the context of hyperglycemia, when the amount of filtered glucose exceeds the capacity of both SGLT1 and SGLT2, the excess amounts of glucose that aren't reabsorbed get excreted into the urine, which is a key characteristic of the diabetic phenotype.

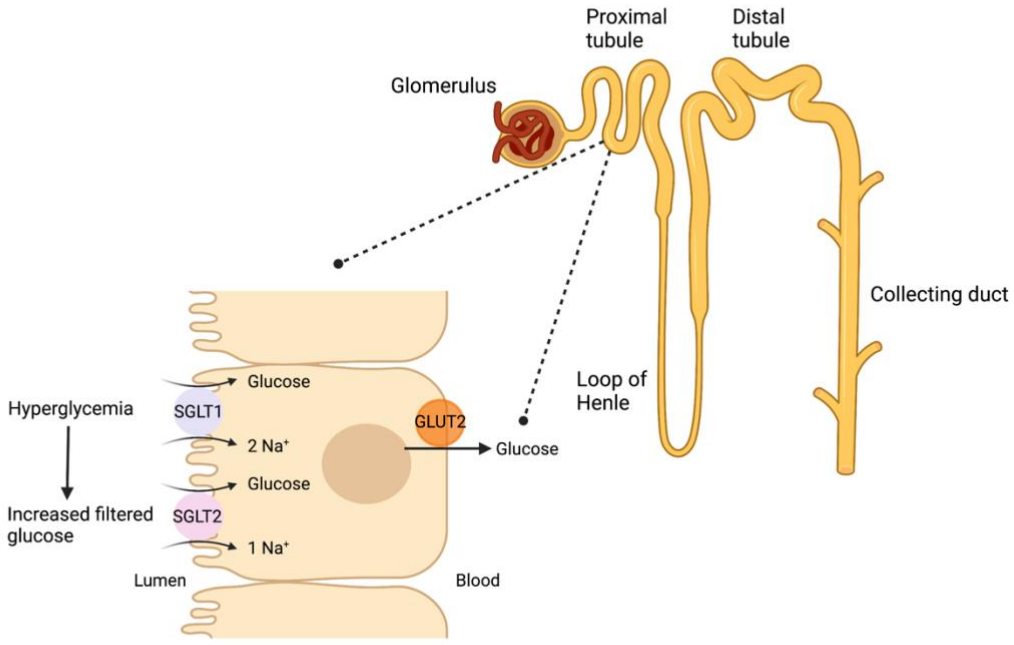


Figure 1.2. Glucose reabsorption in the proximal tubule. Hyperglycemia resulting from diabetes exposes the renal proximal tubule to increasing concentrations of

glucose, leading to hyperabsorption and ultimately proximal tubular dysfunction. Na+ and glucose are filtered on the luminal side by sodium-glucose cotransporter 1 and 2 (SGLT1/SGLT2). Glucose enters back into the blood through facilitated diffusion by GLUT2.

The early phase of diabetes is characterized by an initial increase in glomerular filtration rate (GFR). The normal kidney filters ~120ml/min/1.73 m^2, and GFR can increase to ~166ml/min/1.73 m^2 during the initial phase of hyperfiltration (49). It is generally acknowledged that hyperfiltration is a preceding event to albuminuria and an eventual decline in renal function. Glomerular hyperfiltration leads to hyper-reabsorption of glucose, NaCl and ions, which in turn affects proximal tubular function. This process enhances glucose reabsorption and thereby increasing the expression of SGLT2 which also increases its capacity for reabsorption (44, 50).

In addition to hyper-reabsorption, early glomerular hyperfiltration also contributes to proximal tubular growth. This early prolific phase is initiated early in diabetes and is characterized by increased cellularity, hyperplasia. Hyperplasia is regulated by numerous growth factor signaling pathways, including insulin-like growth factor 1 (IGF-1), platelet derived growth factor (PDGF), epidermal growth factor (EGF) and vascular epidermal growth factor (VEGF) (51). The early period of tubular hyperplasia is subsequently followed by hypertrophy. The tubular hypertrophic phase is mediated by transforming growth factor β1 (TGF-β1) signaling (51, 52), which is initiated by upstream signaling pathways such as JAK/STAT, PKCβ and ERK/p38 (6, 53). As a result of proximal tubule growth, it is also believed that SGLT2 is upregulated as a consequence. Although the

initial phase of hyperglycemia triggers tubular enlargement, this growth is not sustained and is eventually overcome by cellular senescence, fibrosis and tubular interstitial damage (6, 54).

1.3 Treatment of Diabetic Kidney Disease

1.3.1 Targeting the Renin-Angiotensin System

RAS is an important regulator of renal blood volume and vascular resistance. Decreased blood pressure or renal tubular NaCl triggers the activation and release of the proteolytic enzyme renin from juxtaglomerular cells in the renal afferent arterioles. Upon release, renin cleaves its target, angiotensinogen, to produce angiotensin I (Ang I). Ang I is then converted to angiotensin II (Ang II) by angiotensin converting enzyme (ACE). Active Ang II is a potent vasoconstrictor and exerts its effects by binding to Ang II type I receptor (AT1R) and Ang II type II receptor (AT2R). In the proximal tubule, Ang II increases Na-H exchange, leading to increased NaCl reabsorption and increased arterial pressure. Ang II also acts on the adrenal cortex to stimulate the release of aldosterone, which in turn increases NaCl reabsorption. In systemic arterioles, Ang II binds to G protein-coupled receptors (GPCRs), leading to arteriolar vasoconstriction and increasing blood pressure and total NaCl in the blood (55). Hypertension is an early occurrence in diabetes and chronic RAS activation has been indicated as a driver of DKD.

In patients with type 2 diabetic nephropathy (T2DN), lowering blood pressure can slow the progression to ESRD and prevent cardiovascular co-morbidities (56). RAS blockade, which consists of ACE inhibitors and angiotensin receptor blockers (ARBs) work to lower glomerular pressure, vasodilate the renal efferent arterioles, permeability leading to lower

levels of albumin excretion, and are often recommended as the first line of treatment in patients with DKD (57). In 2012, KDIGO (Kidney Disease: Improving Global Outcomes), the standard clinical practice guide for blood pressure management in chronic kidney disease (CKD), recommended RAS inhibitors as the primary treatment for hypertension in CKD. This was based on analyses of clinical trials, which found use of both ACE inhibitors and ARBs reduced kidney failure events (58, 59). In summary, these data suggested that RAS blockade was effective in reducing renal failure in both early and advanced CKD. Since these trials, new data have suggested that there is an initial decline in GFR upon starting treatment with RAS inhibitors, as well as hyperkalemia, and also poses renal and cardiovascular risks in a dose-dependent manner (60). Although RAS inhibitors have been shown to reduce systemic blood pressure and delay the loss of kidney function, their effect on GFR decline poses questions as to whether or not they are an effective treatment for advanced DKD (61).

In addition to its effects on GFR and blood pressure, RAS activation also has effects on mitochondrial function. Ang II can generate mitochondrial ROS and cause dysfunctional mitochondrial metabolism. Studies have shown that antagonism of the AT1R and AT2R can reverse the negative mitochondrial effects induced by Ang II. One study found that treatment of diabetic rats with the AT1 blocker olmesartan restored altered expression of the TCA cycle enzymes citrate synthase and succinate dehydrogenase, as well as the expression of the superoxide generating enzyme NADPH oxidase 2 (Nox2) (62). A separate study using STZ-induced diabetic rats, treatment with the AT1 blocker losartan provided protection in renal mitochondria from changes in mtMP, H2O2 and pyruvate (63). Additionally, a study by Zhu et al. showed that treatment of

podocytes with the mitochondrial antioxidant mitoquinone provided protection from Ang II-induced mitochondrial dysfunction (64). These studies provide evidence that restoring mitochondrial function in models of DKD has a beneficial therapeutic effect on renal outcomes and are examples of drugs that improve kidney function in DKD while simultaneously working to improve mitochondrial function.

1.3.2 Glycemic Control

SGLT2 inhibitors are a new class of diabetic medications that reduce glucose reabsorption in the proximal tubule and promote its urinary excretion, thereby lowering blood glucose levels. During the process of renal glucose reabsorption, glucose is taken up into the cell by SGLT2 and SGLT1. SGLT2 uses one Na^+ ion per one glucose molecule, whereas SGLT1 in the later segments uses two Na^+ ions per one molecule of glucose (65) (Fig. 2). SGLT2 and SGLT1 are responsible for ~97% and 3% of glucose reabsorption, respectively. In response to hyperglycemia, SGLT2 expression is upregulated to compensate for the excess amounts of filtered glucose (66). Since SGLT2 is the main driver of glucose uptake in the proximal tubule, this transporter has been the primary target of sodium-glucose transporter inhibition.

SGLT2 inhibitors were originally developed as a treatment for type 2 diabetes mellitus (T2DM) and were later determined to have kidney and cardiovascular benefits. In 2020, results from the Dapagliflozin and Prevention of Adverse Outcomes in Chronic Kidney Disease (DAPA-CKD) trial showed that the SGLT2 inhibitor dapagliflozin conferred both cardiovascular and kidney benefits among patients with CKD, as defined by reduced estimated GFR, lower risk of ESRD, as well as death as a result of cardiovascular

implications (67). In addition, the Empagliflozin Outcome Trial in Patients with Chronic Heart Failure with Reduced Ejection Fraction (EMPEROR-Reduced), a trial where randomized 3,370 patients with class II, III or IV heart failure and receiving empagliflozin or placebo, showed reduced GFR over time, reduced number of hospitalizations and lower risk of renal outcomes regardless of CKD status (68, 69). The Canagliflozin and Renal Events in Diabetes with Established Nephropathy Clinical Evaluation (CREDENCE) trial in 2019 evaluated the effect of canagliflozin on renal and cardiovascular outcomes in patients with CKD. Results from this trial showed a lower relative risk of ESRD by canagliflozin, lower risk of cardiovascular death, myocardial infarction, stroke and hospitalization due to heart failure (70). Cumulatively, these trials demonstrate the beneficial effects of SGLT2 inhibitors on renal and cardiovascular function in patients with CKD.

Since the aforementioned clinical trials began in 2019, researchers have begun to further investigate the effects of SGLT2 inhibitors in animal models of DKD and the molecular mechanisms underlying their effects on renal function. A study by Hudkins et al investigated the effect of empagliflozin treatment on the pathologic changes in DKD in diabetic BTBR ob/ob mice. This study found that empagliflozin reversed hallmark features of DKD including hyperglycemia, proteinuria, serum creatinine and mesangial matrix expansion (71). Empagliflozin treatment also restored podocyte health, as measured by podocyte number and degree of foot process effacement. The authors suggest that these changes are likely mediated by a reduction in ROS within podocytes and a reduction in pro-inflammatory markers in the renal cortex. A separate study that investigated the effect of dapagliflozin on DKD in mice found a reduction in vascular endothelial damage (72).

Furthermore, the authors showed that dapagliflozin rescued mitochondrial membrane potential, energy metabolism and MB. Dapagliflozin was further shown to have these stimulatory effects on mitochondrial function by activating the SIRT1/PGC1α pathway. These data show that SGLT2 inhibitors have pleiotropic effects in their ability to reduce blood glucose and improve complications associated with DKD.

1.3.3 Insulin Therapy in Glucose Management

Management of T2DM often relies on insulin therapy as a glucose-lowering strategy. Endogenous insulin is produced in the pancreas and is filtered through the glomerulus and is metabolized in proximal tubular cells (73). Through receptor binding, insulin stimulates glucose reabsorption in the proximal tubule (74). Insulin therapy consists of either short-acting insulin analogues (lispro, aspart and glulisine) which confers rapid absorption or long-acting insulin analogues (glargine and detemir) with delayed absorption (75-79). Intensive insulin therapy (multiple injections/day) has been demonstrated as an effective strategy for improving glycemic control in patients with T2DM (80-82). A study evaluating the effect of short-acting insulin on mitochondria in brain tissue of diabetic rats showed that phosphorylation of Drp1 was increased in diabetic rats (83). Treatment with insulin restored Drp1 phosphorylation to nondiabetic control levels. Interestingly, the authors found a significant increase in mitochondrial DNA copy number in diabetic animals, which trended to normalize with insulin treatment. Furthermore, this study showed that insulin treatment significantly reduced the autophagy marker LC3II. Although insulin therapy has been used in DKD patients to improve

glycemic control, it poses the risk of hypoglycemia and importantly, its therapeutic potential in later, more progressed DKD remains unknown.

1.3.4 Lifestyle Modifications

Aside from pharmacological intervention, lifestyle modifications have been extensively documented in DKD management. A number of factors play into improving the complications associated with DKD, including weight management, salt intake, exercise and alcohol intake (84-87). These studies demonstrate that reducing weight can lower blood pressure and initiate a reduction in proteinuria in patients with CKD. Trials assessing weight reduction evaluated multiple factors and showed that dietary modification consisting of increased fruit and vegetable intake as well as salt reduction in overweight individuals was demonstrated to lower blood pressure (88). In addition to dietary modification, daily exercise is also recommended in patients with CKD (57).

The beneficial effects of exercise on blood pressure have been widely studied. It is also important to note that exercise has been shown to stimulate mitochondrial function (89). The increased demand for oxidative capacity during exercise has an overall stimulatory effect on mitochondrial function and importantly, studies have shown that PGC1α is activated following exercise (90, 91). Following activation, PGC1α initiates transcription of genes encoding mitochondrial proteins, mitochondrial DNA and oxidative metabolism, ultimately leading to the induction of MB (92). Besides MB, it has also been shown that exercise leads to increased mitochondrial mass and mitochondrial enzyme efficiency (93). Specifically, aerobic exercise increases activity of the ETC enzymes succinate dehydrogenase, NADH dehydrogenase, NADH cytochrome-c reductase and

cytochrome c oxidase (94). Importantly, these findings have been replicated in humans (95, 96). These findings demonstrate yet another example of how inducing MB and improving mitochondrial function can lead to the improvement of morbidities associated with DKD.

1.4 Pharmacological Targeting of Mitochondrial Biogenesis and Dynamics

1.4.1 Induction of Mitochondrial Biogenesis

Over the past years, studies have provided evidence that decreased MB and PGC1α are important components in the development and progression of DKD. The study by Lee et al which demonstrated that activation of proximal tubule PGC1α had a beneficial effect on renal function, supports this hypothesis. In conjunction with this study, it has also been shown that SIRT1, an upstream activator of PGC1α was downregulated in podocytes and glomeruli in human diabetic kidneys as well as in diabetic mice (97, 98). Furthermore, selective pharmacological activation of SIRT1 in cultured podocytes increased SIRT1-mediated PGC1α activity and protected the cells from mitochondrial dysfunction resulting from hyperglycemic injury (99). When the SIRT1 agonist BF175 was given in diabetic mice, significant improvements in albuminuria and glomerular injury were observed. Importantly, a study analyzing cortical tubulointerstitial samples in kidney biopsy samples from patients with DKD found that PGC1α mRNA expression was decreased when compared to healthy controls (100), indicating that the alterations in PGC1α and mitochondria are also present in clinically relevant patients. Together, these studies support the idea that the loss of PGC1α contributes to worsened renal function and that induction of MB can alleviate the some of the pathophysiological components of DKD.

The antihyperglycemic agent metformin has been used over the past 50 years in patients with T2DM (101). The mechanism of action of metformin is one that has been classified as “different” from other antihyperglycemic agents in its ability to lower blood glucose levels. Metformin decreases both hepatic gluconeogenesis and intestinal absorption of glucose, and improves insulin sensitivity (102). At the molecular level, metformin specifically inhibits mitochondrial ETC complex I. By inhibiting complex I function, this leads to an increased AMP/ATP ratio and activation of AMPK, which has been shown to lead to increased PGC1α activation (103, 104). Metformin has been shown to induce MB and increase carbonyl cyanide p-trifluoro-methoxyphenyl hydrazone-oxygen consumption rates (FCCP-OCR) in renal proximal tubule cells (RPTC) (105, 106).

Identification of compounds that stimulate PGC1α activity and expression as potential mitochondrial therapeutics has been a developing topic of interest. Our laboratory developed a high-throughput screening assay to measure maximal mitochondrial respiration as a marker of MB (105). This screening assay relied on the use of RPTC grown under improved culture conditions, which are highly dependent on aerobic respiration, and subjected to Seahorse XF96 analysis of FCCP-uncoupled OCR (40, 105, 107). By measuring OCR following FCCP, we can observe early dysfunction of the electron transport chain. Higher OCR following pharmacological treatment indicates the generation of new mitochondria or more efficient existing mitochondria. Using this method, we identified several hit compounds as inducers of MB. Among these compounds, we identified the β2-AR agonist formoterol and the 5-hydroxytryptamine-1F receptor (5-HT1FR) agonists LY344864 and LY334370, which were later determined to

be stimulators of PGC1α. Studies from our laboratory have shown that activation of PGC1α leads to MB and promotes recovery from both acute and chronic diseases (108-113). Our recent study showed that treatment of diabetic mice and RPTC exposed to high glucose had mitochondrial bioenergetic and dynamics profiles that were similarly altered and that treatment with formoterol reversed these effects (19), indicating that in addition to its ability to induce PGC1α and MB, formoterol also regulates mitochondrial dynamics and bioenergetics, although these signaling pathways have not yet been established. These studies support the idea that pharmacologically targeting mitochondria in renal diseases can be a promising therapeutic strategy.

1.4.2 Targeting Mitochondrial Dynamics

Targeting other facets of mitochondrial dysfunction such as mitochondrial dynamics has been shown to improve of hallmark features of DKD. The study from Ayanga et al which demonstrated that pharmacological inhibition of Drp1 with Mdivi-1 in podocytes had a multitude of beneficial effects. Treatment with Mdivi-1 significantly reduced albumin:creatinine ratio, mesangial matrix expansion, glomerular basement membrane thickening, attenuation of podocyte foot process effacement and restored the mitochondrial phenotype to that of non-diabetic controls. These findings provide evidence that improving mitochondrial function can protect against DKD progression.

We showed that proteins involved in mitochondrial dynamics were altered in diabetic mice and in RPTC exposed to high glucose, favoring a pro-fission mitochondrial state (19). In both models, treatment with the β2-AR agonist formoterol pharmacologically restored altered expression of mitochondrial dynamics proteins and improved

mitochondrial function. In addition to this study, others have demonstrated that inhibition of mitochondrial fission with the natural compound berberine in diabetic db/db mice significantly reversed elevated glucose, podocyte damage and mesangial matrix expansion by abolishing Drp1 activation (36). Separate studies evaluating podocytes in diabetic mice showed that podocyte-specific deletion of Drp1 resulted in reduced podocyte damage and prevention of albuminuria (114). Cumulatively, these results demonstrate that inhibition of mitochondrial dynamics by specifically targeting Drp1 can promote mitochondrial homeostasis and partially restore renal function. An effort to elucidate the signaling pathways associated with altered mitochondrial dynamics will allow for a better understanding of how mitochondria become dysfunctional in the early phase of hyperglycemia.

1.4.3 Agonism of the β2-Adrenergic Receptor and Renal Mitochondrial Function

It has been established that β2-ARs are expressed in the kidney and play various important roles in renal physiology such as regulation of glomerular filtration and renal blood flow, NaCl reabsorption and renin secretion (115-117). Formoterol is a long-acting β2-AR agonist with high selectivity and affinity for the β2 subtype (K_D=-8.63±0.02) and is approved for the treatment of asthma (118). Our laboratory has shown that formoterol improves renal function in ischemia/reperfusion (IR)-induced acute kidney injury (AKI) by activating proximal tubule β2-AR to induce MB (111). However, clenbuterol, another β2-AR agonist was shown to have an inhibitory effect on MB through suppression of PGC1α and by activating receptor-interacting protein 140 (RIP140), a negative regulator of MB (119). In addition, when a panel of β2-AR agonists were tested for their ability to induce

MB, not all agonists had the same effect: some yielded partial MB and some non-MB (120) (Table 1). Interestingly, the ability of these agonists to induce MB did not correlate with receptor affinity or selectivity. These contrasting results among agonists suggests that the mitochondrial biogenic effect of β2-AR agonists has some sort of functional selectivity. A recent study from our laboratory elucidating the signaling pathway leading to MB by formoterol demonstrated that upon β2-AR binding, formoterol activates Gβγ/Akt/eNOS/sGC pathway to induce MB (121) (Fig. 3). This signaling pathway was not induced by the selective β2-AR agonist clenbuterol.

No MB	Partial MB	Full MB
Epinephrine	Terbutaline	Formoterol
Norepinephrine	Metaproterenol	Fenoterol
Isoproterenol	Ritodrine	Procaterol
Isoetharine	Cyclopentobutaneprine	Atomoxetine
Clenbuterol		Nisoxetine

Table 1. Mitochondrial biogenic effects of β2-AR agonists. Agonists are classified in terms of their ability to induce mitochondrial biogenesis (MB) yielding compounds with no MB, partial MB or full MB.

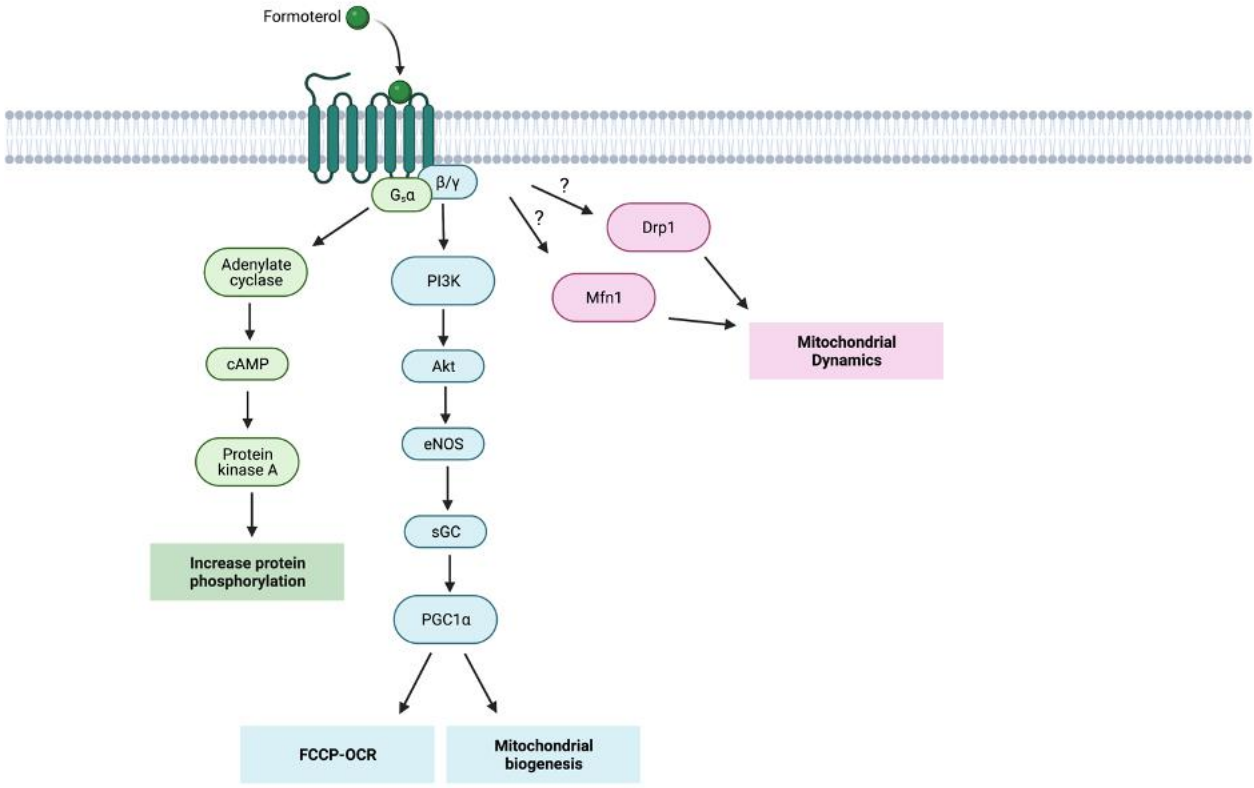


Figure 1.3. Signaling mechanisms/pathways of β2-AR activation by formoterol.

Through Gαs, formoterol induces a classical β2-AR signaling pathway through activation of adenylate cyclase (AC), leading to increased cAMP production, protein kinase A (PKA) activation and increased phosphorylation of substrates. Formoterol also activates signaling through Gβγ. Activation of Gβγ leads to phosphatydilinositol 3 kinase (PI3K), leading to protein kinase B (PKB/Akt) phosphorylation and activation of

endothelial nitric oxide synthase (eNOS), soluble guanylyl cyclase (sGC) and peroxisome proliferator-activated receptor gamma coactivator (PGC1α) and ultimately leading to induction of mitochondrial biogenesis. Formoterol regulates mitochondrial dynamics through undetermined signaling pathways.

More recently, we demonstrated that formoterol treatment improved podocyte recovery from glomerular injury by promoting MB and increasing ETC protein levels (122). It has also been shown that β2-AR agonists are potent inhibitors of phorbol myristate acetate (PMA) induced tumor necrosis factor α (TNFα) production in macrophages (123). This finding led the authors to further investigate the anti-inflammatory effects of β2-AR agonists in STZ-induced diabetic rats. It was found that the β2-AR agonists metaproterenol and salbutamol inhibited TNFα production that was induced by the diabetic phenotype. These effects were dependent on β-arrestin2/NFκB signaling. The authors further demonstrated that salbutamol treatment of diabetic rats reduced collagen production and attenuated TNFα as well as other inflammatory markers. These results suggest that in addition to their mitochondrial effects, β2-AR agonists also work to improve the diabetic phenotype by regulating monocyte/macrophage activation. Thus, there is evidence that activation of the β2-AR by agonists including formoterol not only improves mitochondrial function in several models of kidney injury, but also through multiple and distinct signaling pathways. These data support the idea that β2-ARs have the potential to be novel therapeutic targets in renal disease including DKD.

1.5 Conclusions

DKD is a complex, multifaceted disease that affects a variety of cellular and mitochondrial processes. In this review, we identify aspects of mitochondrial dysfunction, particularly in the renal proximal tubule and in glomerular podocytes that take place in the early stages of hyperglycemia, ultimately leading to disease progression. Further, we discuss pharmacological therapeutics to treat DKD and their effect on mitochondrial function. Hyperglycemia leads to early glomerular hyperfiltration, exposing the proximal to increased amounts of glucose. This physiological process triggers proximal tubular and glomerular dysfunction through a number of molecular and pathophysiological processes including tubular growth and fibrosis. However, because the proximal tubules are rich in mitochondria and require an abundance of energy, mitochondrial dysfunction plays an important role in declining renal function. Existing therapies to treat DKD primarily consist of targeting blood pressure through RAS inhibition and glycemic control via SGLT2 inhibition. Specifically, SGLT2 inhibitors have shown to be a promising treatment for DKD due their pleiotropic benefits not only on the kidney, but also on the cardiovascular system. A common property of these existing therapies lies within their ability to modulate mitochondrial function. Interestingly, RAS inhibition, SGLT2 inhibition and lifestyle modification through exercise appear to have beneficial renal effects at least in part through restoring mitochondrial function either by inducing MB or restoring the imbalance in mitochondrial dynamics. Although these drugs have effects on mitochondrial function,

there are no existing compounds that specifically target mitochondrial function to treat DKD.

Despite the fact that emerging therapies for DKD such as SGLT2 inhibitors have shown to be promising therapeutics, there is a need for the identification and development of new therapies to treat DKD. To this end, specifically targeting mitochondrial dysfunction in the early stages of hyperglycemia could be a pivotal strategy in targeting renal dysfunction. Thus, understanding whether the very early mitochondrial responses in diabetes could potentially lead to new insights about the initial responses that drive later structure and functional abnormalities in DKD. Existing FDA-approved compounds such as formoterol have been demonstrated to modulate mitochondrial function in some way, either through induction of MB or targeting mitochondrial dynamics. The specific Drp1 inhibitor Mdivi-1 was shown to have a number of benefits not only on glomerular podocyte function, but also on key elements of the diabetic phenotype, such as albuminuria and blood glucose levels (2).

Our laboratory has extensively studied the effects of the β2-AR agonist formoterol on both acute and chronic renal diseases. We have reported that formoterol promotes renal function by activating PGC1α and inducing MB, as well as through targeting the mitochondrial dynamics proteins Drp1 and Mfn1 through novel and distinct mechanisms. Further elucidation of these mechanisms will allow for a better understanding of the diverse ways in which formoterol modulates mitochondrial function. Identification of the proteins involved in disrupted mitochondrial dynamics will allow for the development of drugs to target specific steps in these altered signaling pathways. As summarized in figure

1.4, we suggest that repurposing FDA-approved therapeutics that restore mitochondrial function, such as formoterol, may be beneficial for the treatment of DKD.

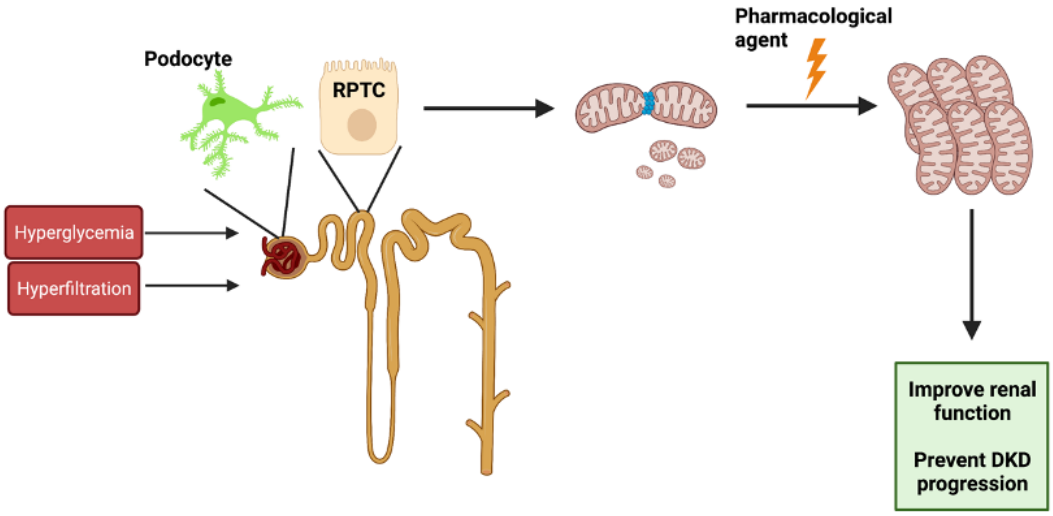


Figure 1.4. Restoring mitochondrial function in hyperglycemia improves renal function and prevents disease progression. Hyperglycemia and hyperfiltration lead to glomerular and tubular damage (i.e. podocytes and renal proximal tubule cells) and is characterized by renal mitochondrial dysfunction. Pharmacological restoration of mitochondrial function can improve renal function and prevent progression of DKD.

Chapter 2:

Regulation of mitochondrial dynamics and energetics in the diabetic renal proximal tubule by the β2-adrenergic receptor agonist formoterol

Text and figures derived from Cleveland, K.H, et al. (2020). *Am J Physiol Renal Physiol*, 319(5): F773-F779.

2.1 Abstract

Diabetes is a prevalent metabolic disease that contributes to ~50% of all end stage renal diseases (ESRD) and has limited treatment options. We previously demonstrated that the β_2-adrenergic receptor (AR) agonist formoterol induced mitochondrial biogenesis and promoted recovery from acute kidney injury. Here, we assessed the effects of formoterol on mitochondrial dysfunction and dynamics in renal proximal tubule cells (RPTC) treated with high glucose and in a mouse model of type 2 diabetes. RPTC exposed to glucose exhibited increased electron transport chain (ETC) complex I, II, III and V protein levels, reduced ATP levels and uncoupled oxygen consumption rate (OCR) compared to RPTC cultured in the absence of glucose or osmotic controls. ETC proteins, ATP and OCR were restored in RPTC treated with formoterol. High-glucose RPTC exhibited an increase in phospho-Drp1, a mitochondrial fission protein, and a decrease in Mfn1, a mitochondrial fusion protein. Diabetic db/db and non-diabetic db/m mice were treated with formoterol or vehicle for 3 weeks and euthanized. Db/db mice showed increased renal cortical ETC protein levels in complexes I, III and V and decreased ATP and these changes were prevented by formoterol. Phospho-Drp1 was increased and Mfn1 was decreased in db/db mice and formoterol restored both to controls levels.

Together, these findings demonstrate that hyperglycemic conditions *in vivo* and exposure of RPTC to high glucose similarly alter mitochondrial bioenergetic and dynamics profiles, and that treatment with formoterol can reverse these effects. Formoterol may be a promising strategy for treating early stages of DKD.

2.2 Introduction

Diabetic kidney disease (DKD) is the leading cause of end stage renal disease (ESRD) in the United States and many other countries (124). DKD occurs through a variety of pathogenic processes that are in part driven by hyperglycemia and glomerular hypertension (125-127), that leads to gradual loss of kidney function and eventually progresses to ESRD.

In the early phase of DKD, hyperglycemic conditions affect mitochondrial dynamics and energy generation, leading to mitochondrial dysfunction (128, 129). Mitochondria continuously undergo fission and fusion, mitochondrial dynamics, in response to cellular and environmental signals to maintain a balanced mitochondrial homeostasis (1, 130). Studies demonstrate that mitochondrial dynamics are altered in mouse models of DKD and that restoring the balance between fission/fusion can prevent the progression of DKD (131). Mitochondrial fission is primarily mediated by Drp1 (dynamin related protein 1), a dynamin-related GTPase. In response to a stimulus, Drp1 is phosphorylated and translocates to the mitochondria where it forms ring-like oligomers around the mitochondrial membrane to initiate fission (1). One of the characteristic features of mitochondrial dysfunction in DKD is an excess of mitochondrial fission (2, 3, 41, 132). In both *in vitro* and *in vivo* models of DKD, Drp1 phosphorylation is elevated and pharmacological inhibition of Drp1 phosphorylation results in decreased progression of DKD (1, 2). In contrast, mitochondrial fusion is mainly mediated by mitofusins 1 and 2 (Mfn1/2). During fusion, Mfn1/2 together provide a docking function to promote fusion of the outer membranes of adjacent mitochondria through GTP-mediated hydrolysis (133).

Despite the fact that much attention has been given to DKD as a glomerulopathy, there is evidence that the proximal tubule plays an important role in the pathogenesis of this disease (4, 134). Proximal tubules are the major energy consuming cells in the second most energetically demanding organ in the body and are rich in mitochondria (9, 41). Proximal tubule function and health are particularly susceptible to mitochondrial dysfunction. Studies have shown that expression of proteins in electron transport chain (ETC) complexes I-V and function of these complexes is decreased in models of DKD (42, 135) leading to reduced mitochondrial oxygen consumption rates (OCR) and ATP production (42).

Our laboratory developed a phenotypic assay to measure mitochondrial biogenesis (MB) and identified the β_2-adrenergic receptor (AR) agonist formoterol as a potent MB inducer (105, 136, 137). Activation of the β_2-AR by formoterol promotes recovery from acute kidney injury (AKI) by inducing MB in *in vivo* and *in vitro* models of kidney injury (111, 137). Furthermore, our laboratory has shown that formoterol enhances podocyte recovery from injury in a mouse model of focal segmental glomerular sclerosis (122). The goal of this study was to determine if formoterol treatment in an early stage mouse model of DKD restores the altered expression of mitochondrial dynamics and energetics in renal proximal tubules and in renal proximal tubule cells (RPTC) treated with high glucose.

2.3 Methods

2.3.1 Animal experiments

Male BKS.Cg-*Dock7*^{m} +/+ *Lepr*^{db}/J mice were obtained from The Jackson Laboratory (Bar Harbor, NE). Mice homozygous (db/db) and heterozygous (db/m) for the Lepr^{db} mutation were housed in a room at a constant temperature of 22°C±2°C with 12 hour light-dark cycles. Db/m and db/db mice 10 weeks of age were treated with vehicle (0.1% DMSO) or formoterol (1 mg/kg) via intraperitoneal (i.p.) injection daily for three weeks. At 13 weeks, mice were euthanized and kidneys were harvested. All studies were approved by The University of Arizona in accordance with the guidelines set forth by the National Institute of Health Guide for the Care and Use of Laboratory Animals.

2.3.2 Serum glucose measurements

Blood was collected from diabetic db/db mice and db/m control mice aged 10, 11, 12 and 13 weeks of age. Glucose levels were measured using a glucose colorimetric detection kit (ThermoFisher) according to the manufacturer's protocol.

2.3.3 In vitro studies

Female New Zealand White rabbits (1.8-2 kg) were purchased from Charles River (Oakwood, MI/Canada). RPTC were isolated using the iron oxide perfusion method and grown in 35-mm tissue culture dishes under improved culture conditions similar to what is observed *in vivo* (40). The culture medium was a 1:1 mixture of Dulbecco's modified Eagles medium /F-12 (without glucose, phenol red or sodium pyruvate) supplemented

with 15mM HEPES buffer, 2.5mM L-glutamine, 1μM pyroxidine HCl, 15mM sodium bicarbonate and 6 mM lactate. Hydrocortisone (50nM), selenium (5ng/ml), human transferrin (5μg/ml), bovine insulin (10nM) and L-ascorbic acid-2-phosphate (50μM) were added to fresh culture medium. Cells grown in the presence of glucose were supplemented with 17mM D-glucose or 17mM D-mannitol (osmotic control). Confluent RPTC were used for all experiments.

2.3.4 Immunoblot analysis

Protein was extracted from mouse renal cortical tissue and RPTC cultures using RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, pH 7.4). Protease inhibitor cocktail (1:100), 1mM sodium fluoride and 1mM sodium orthovanadate (Sigma-Aldrich, St. Louis, MO) were added fresh before each extraction. Equal protein quantities (10μg) were loaded onto 4-15% SDS-PAGE gels, resolved by gel electrophoresis and transferred onto nitrocellulose or PVDF membranes (Bio-Rad). Membranes were blocked with 5% nonfat milk in TBST and incubated overnight with primary antibody at 4 °C with agitation. Primary antibodies used in these studies include p-Drp1 (1:1000) (ab#193216), Mfn1 (1:100) (ab#104274), Mfn2 (1:1000) (ab#56889) and total oxphos rodent WB antibody cocktail (1:1000) (ab110413) all from Abcam; Histone H3 (1:1000) (#4499) and GAPDH (1:1000) (#5174) were all purchased from Cell Signaling Technology (Danvers, MA). Drp1 (1:1000) (#32898) was purchased from Santa Cruz Biotechnology (Dallas, TX). Membranes were incubated with the appropriate horseradish peroxidase conjugated secondary antibody before visualization using

enhanced chemiluminescence (Thermo Scientific, Waltham, MA) and the GE ImageQuant LAS4000 (GE Life Sciences, Marlborough, MA). Optical density was determined using Bio-Rad Image Lab 6.0.

2.3.5 ATP measurements

ATP levels of Confluent RPTC cultures were measured using the CellTiter-Glo Luminescent Cell Viability Assay Kit (Promega, Madison, WI) and the manufacturer's protocol.

2.3.6 Analysis of oxygen consumption

Cultured RPTC were grown on 96-well XF96 extracellular flux analyzer plates (Seahorse Bioscience, Billerica, MA) at a cell density of $1.6x10^4$ cells per well and grown in media containing 0mM glucose, 17mM mannitol or 17mM glucose for 96 hr. Basal OCR was measured three times using the Seahorse Bioscience XF96e Analyzer before injection of carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) (2μM), rotenone (0.5μM) or antimycin A (0.5μM) (Sigma Aldrich) to measure OCR as previously described (105). OCR was reported as picomoles per minute, and the results were normalized as a percentage of vehicle control (DMSO).

2.3.7 Statistical analysis

All data are shown as mean±SEM. One-way or two-way analysis of variance followed by Tukey's post hoc test was performed for comparisons of multiple groups. $P<0.05$ was

considered statistically significant. All statistical tests were performed using the GraphPad Prism software (GraphPad Software, San Diego, CA).

2.4 Results

2.4.1 Formoterol restores mitochondrial energetics and dynamics proteins in high glucose treated RPTC

To evaluate the effects of high glucose exposure on RPTC, we grew primary cultures of RPTC in defined media without serum and growth factors, and in no glucose, 17mM mannitol (osmotic control) or 17mM glucose co-treated with either vehicle or formoterol for 24, 48, 72 or 96 hr. No changes in ATP were observed at 24, 48 and 72 hr in any group (data not shown). At 96 hr, RPTC grown in the absence of glucose or with mannitol had no effect on ATP levels (Figure 2.1A). However, vehicle treated RPTC grown in high glucose decreased ATP by 30% and treatment with formoterol restored ATP to control levels.

Studies evaluating mitochondrial energetics in the context of DKD show that high glucose changes expression of ETC proteins (42, 138). To investigate whether high glucose leads to changes in ETC protein expression in RPTC, we measured ETC subunits representing different complexes in high glucose co-treated with vehicle or formoterol for 96 hr. RPTC grown in high glucose exhibited increased ETC complex V, III and I protein expression (Figure 2.1B-G). Formoterol treatment restored complex V and III, trended to reduce complex I protein expression to control levels, and had no effect on the expression of ETC complexes IV and II.

To test whether changes in RPTC ETC proteins leads to functional changes in ETC complex activity, we used the Seahorse XF96 Analyzer to measure parameters of mitochondrial function including maximal respiration and complex I and III function. RPTC grown in 0mM glucose or 17mM mannitol controls and treated with vehicle had no effect on maximal respiration, and treatment with formoterol increased FCCP-OCR, a measure of maximal ETC activity, (Figure 2.2A). High glucose decreased FCCP-uncoupled OCR compared to controls. Treatment with formoterol increased maximal respiration to greater than control levels. To measure complex III function, we injected antimycin A (AA) to inhibit complex III activity and measured OCR. RPTC grown in high glucose had decreased OCR after AA injection compared to controls, and co-treatment with formoterol restored OCR to control levels (Figure 2.2B). Injection of the complex I inhibitor rotenone had no effect on OCR in RPTC (Figure 2.2C).

To test the effects of glucose and formoterol on mitochondrial dynamics proteins, we grew RPTC in high glucose co-treated with either vehicle or formoterol for 96 hr. High glucose increased phosphorylation of Drp1 at S637 compared to controls (Figure 2.2D and E). RPTC co-treated with formoterol prevented the increase in Drp1 phosphorylation. In contrast, high glucose decreased expression of the mitochondrial fusion protein Mfn1, and co-treatment with formoterol restored Mfn1 expression (Figure 2.2F). Glucose had no effect on Mfn2 expression levels (Figure 2.2G).

2.4.2 Formoterol restores mitochondrial energetics proteins in diabetic db/db mice

To determine if formoterol induces a similar preventative effect under *in vivo* conditions, we tested its effect on ETC proteins db/db mice. Db/m and db/db mice aged 10 weeks were treated with vehicle (0.1% DMSO) or formoterol (1 mg/kg) daily for three weeks. Formoterol treatment had no effect on body weight of db/m or db/db mice (Figure 2.3A and B). Db/db mice treated with vehicle had elevated serum glucose compared to controls, and formoterol trended to reduce glucose concentrations (Figure 2.3C and D). Db/db mice had decreased ATP compared to db/m controls, and formoterol prevented the decrease in ATP production (Figure 2.4A). We also tested for changes in proximal tubule ETC proteins in diabetic mice. Vehicle-treated db/db mice had increased ETC complex subunit expression representing complexes I, II, III and V. Formoterol treatment maintained ETC complex I, II and V subunits at the same level as db/m control mice (Figure 2.4B-G).

2.4.3 Formoterol restores mitochondrial dynamics proteins in diabetic db/db mice

To determine if mitochondrial dynamics are altered in diabetic mice, we harvested kidneys from db/m and db/db mice at 13 weeks and measured fission/fusion proteins in renal cortex. Vehicle-treated db/db mice showed elevated phosphorylation of the mitochondrial fission protein Drp1 at S637 (Figure 2.5A and B). Diabetic mice treated with formoterol did not show the same increase in phospho-Drp1. Conversely, Mfn1 was decreased compared to db/m controls, and formoterol treatment maintained Mfn1 expression at control levels (Figure 2.5C). Mfn2 expression was unchanged under all conditions (Figure 2.5D).

2.5 Discussion

This study investigated the long-acting β_2-AR agonist formoterol as a potential therapeutic for preventing mitochondrial dysfunction in early models DKD. β_2-AR activation has been extensively studied in multiple diseases, but has not been explored comprehensively in kidney diseases (139). In the kidney, β_2-ARs regulate a number of physiological functions (115, 116) and its role in the proximal tubule has been investigated in disease models such as AKI, where pharmacological activation of the β_2-AR improved renal function after acute injury. (111, 136). Prior reports of β_2-AR signaling in DKD have focused on its role in inhibition of pro-inflammatory responses particularly in monocytes and macrophages (123). Because of the importance of proximal tubular responses and mitochondrial dysfunction early in the pathogenesis of DKD, we investigated whether β_2-AR signaling improved mitochondrial function in proximal tubules. Here, we report that formoterol maintains mitochondrial dynamics and energetics in the proximal tubule in *in vitro* and *in vivo* models of early DKD.

We previously reported that RPTC treated with 17 mM glucose decreased basal and uncoupled respiration after 96 hr (140). Therefore, we chose 96 hr as the treatment period for all RPTC experiments in this study. In the presence of glucose, phosphorylation of Drp1 at S637 increased and formoterol prevented phosphorylation. In contrast, glucose decreased Mfn1 expression and was restored by formoterol. The mechanism by which formoterol produces these effects is unknown and will be studied in future experiments. Other studies have shown that formoterol promotes recovery from AKI by inhibiting

mitochondrial fission through Drp1 phosphorylation, as well as studies showing that pharmacological inhibition of Drp1 in podocytes prevents the progression of DKD (2, 111). Based on these observations, we suggest that high glucose promotes a pro-fission mitochondrial state in RPTC that is prevented by formoterol.

RPTC are particularly susceptible to mitochondrial dysfunction in diabetes. Studies have demonstrated that ETC complex expression and function in the renal cortex are decreased in mouse DKD models at later time points (42, 135). These changes are accompanied by decreased mitochondrial function as measured by OCR leading to less efficient ATP production (42). However, ETC complex protein expression was elevated in our mouse model of early diabetes (prior to development of pathologic features of DKD) as well as in RPTC grown in high glucose. These differences can be explained by the differences in stages of DKD, where the early increase in ETC complex expression may be driven by increased glucose metabolic flux that has been demonstrated in kidney cortex *in vivo (42)*. Together, our results and those in prior publications suggest that there is an initial increase in oxidative phosphorylation proteins in early diabetes that then reverses during the course of DKD pathologic changes.

Despite the increase in ETC complex protein expression in our model, we found that maximal respiration was decreased in the presence of high glucose along with ETC complex III function. FCCP-OCR predicts how well ETC complexes respond to an injury or environmental stressor. In this study, high glucose levels resulted in decreased RPTC mitochondrial and ETC function. Thus, the observed increases in ETC protein levels in this model could reflect a compensatory response to the reduction in ATP generation that

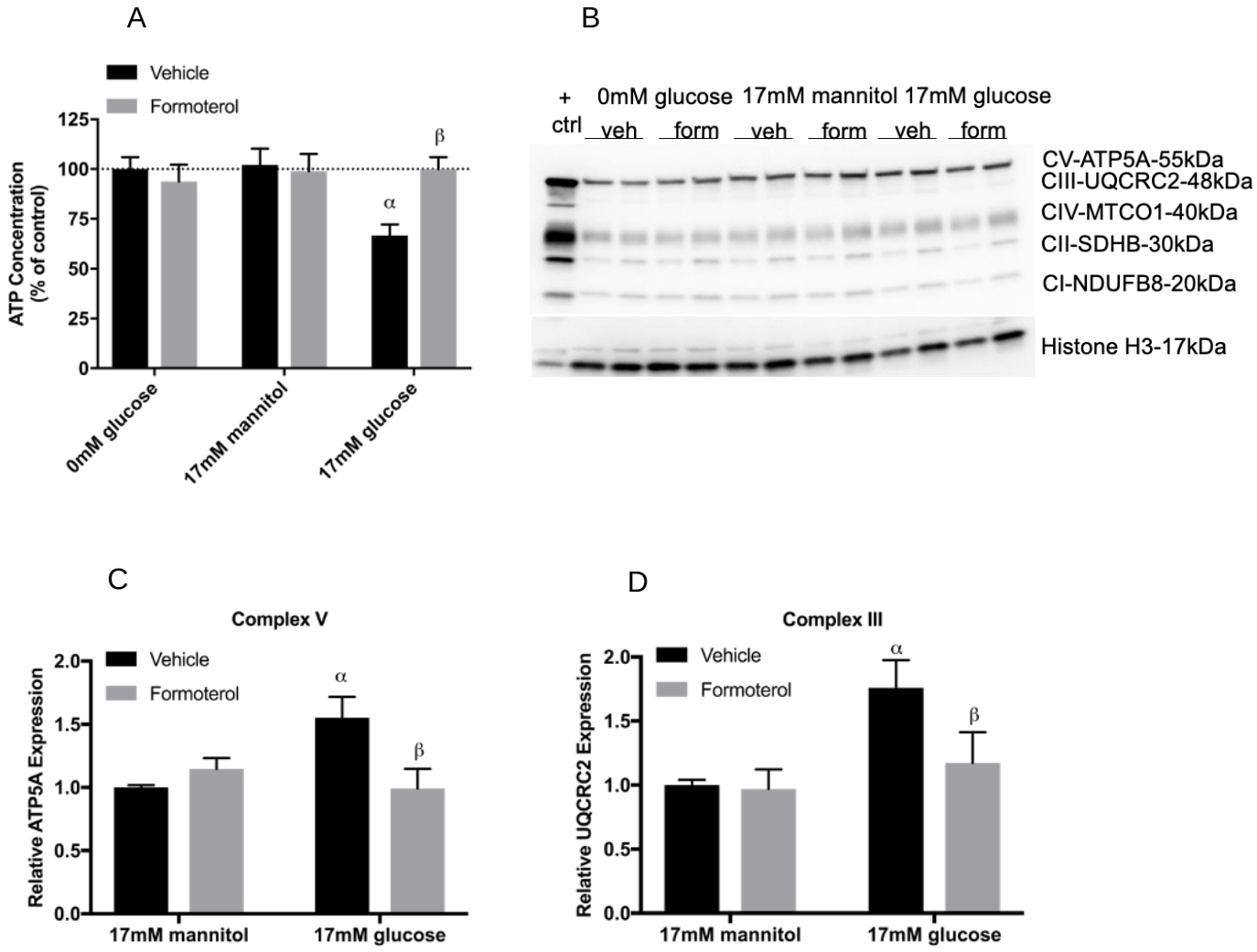
resulted from reduced mitochondrial number or efficiency. Conversely, it is possible that the increase in ETC protein expression itself led to injury and mitochondrial dysfunction which was then reflected in the reduced OCR. Future studies will examine whether the reduction in OCR in diabetes is primary or secondary and to identify the mechanisms by which formoterol works to improve mitochondrial dynamics and energetics in the diabetic renal proximal tubule. This is the first report indicating that pharmacological activation of the β_2-AR by formoterol improves the disrupted mitochondrial dynamics and energetics to regulate renal mitochondrial homeostasis in two early-stage models of DKD. These findings support the clinical relevance for the use of formoterol as a therapeutic agent to prevent the initiation as well as the progression of DKD.

2.6 Conclusions

In this study, we demonstrated that RPTC treated with high glucose had altered expression of proteins involved in mitochondrial energetics and dynamics, further leading to decreased mitochondrial respiration. These findings are summarized in Figure 2.1. These results are in agreement with previous studies from our laboratory, which demonstrated that RPTC exposed to chronic high glucose had decreased expression of calpain 10, an important regulator of mitochondrial homeostasis, and decreased maximal respiration (140). The effects of high glucose on mitochondrial protein expression and function seen in RPTC, were similarly demonstrated in diabetic mice. The observed alterations in mitochondrial protein expression and function were restored upon formoterol treatment, demonstrating that selective agonism of the β2-AR could be a potential therapeutic strategy for restoring mitochondrial function in early

DKD. Elucidation of the signaling mechanisms through which glucose and formoterol regulate phosphorylation of Drp1 and expression of Mfn1 would be of interest in future studies. Identification of the proteins involved in these signaling pathways will allow for 1) a better understanding of the diverse signaling pathways induced by formoterol and 2) specific targeting of proteins involved in mitochondrial dynamics.

2.7 Figures and Figure Legends



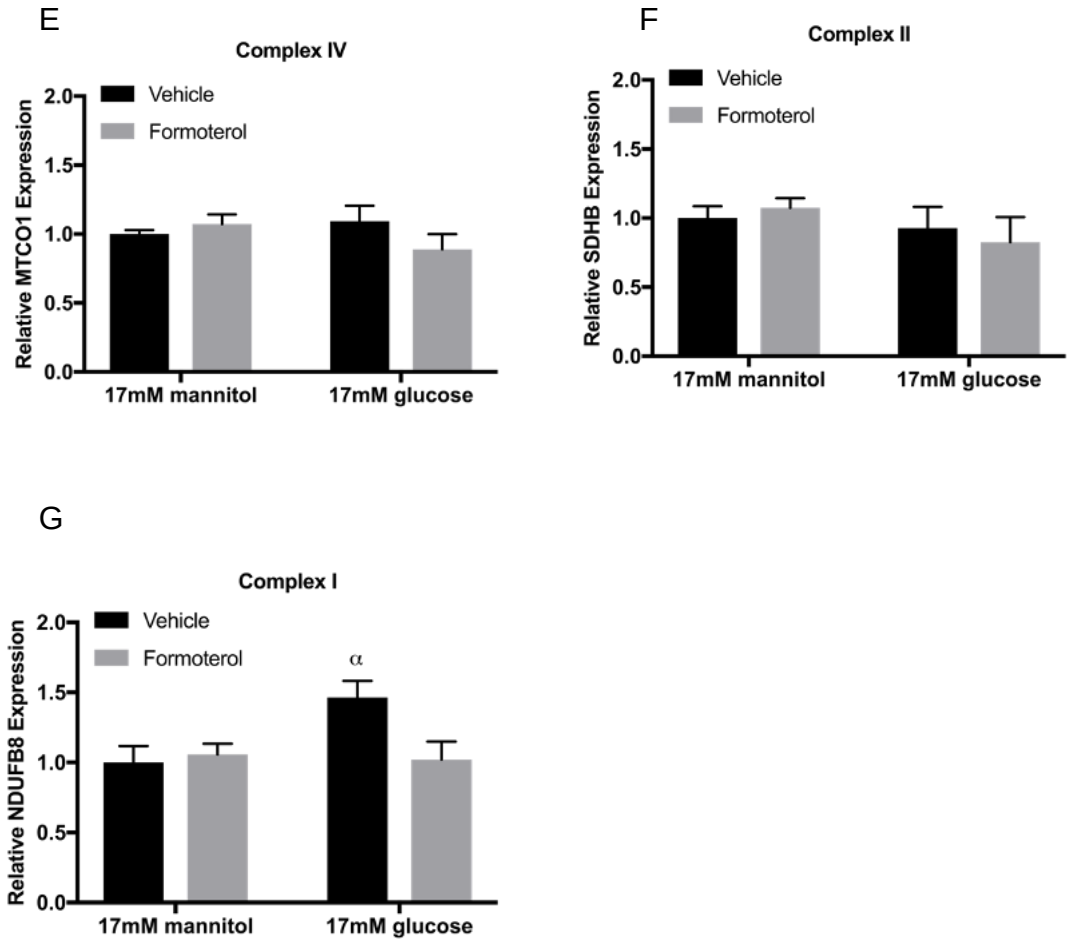
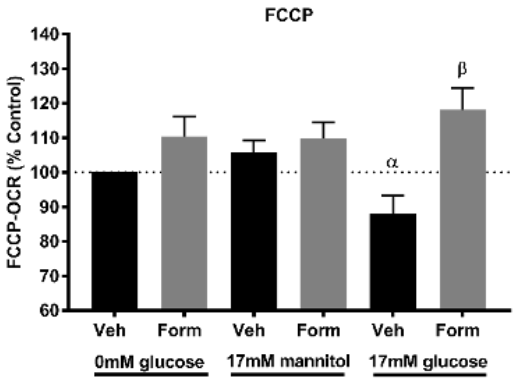
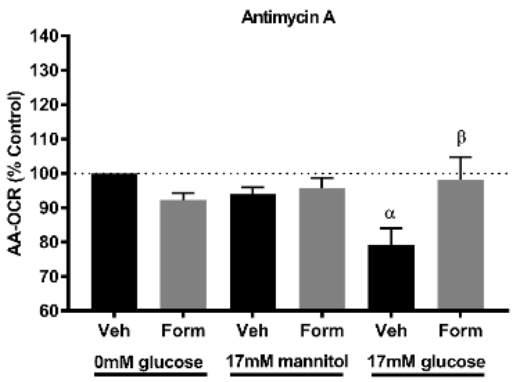


Figure 2.1. **Formoterol restores mitochondrial energetics in glucose-treated RPTC**. RPTC were grown in 0mM glucose, 17mM mannitol or 17mM glucose and co-treated with formoterol (30 nM) for 96 hr and (A) ATP content was measured (B) Immunoblot of total oxphos proteins and densitometry analysis of (C) ATP5A, (D) UQCRC2, (E) MTCO1, (F) SDHB and (G) NDUFB8. Data represented as mean±SEM, n=6-9. Statistical significance was determined by two-way ANOVA and Tukey-Kramer post-hoc test. α=P<0.05 compared to 0mM glucose vehicle, β=P<0.05 compared to 17mM glucose vehicle.

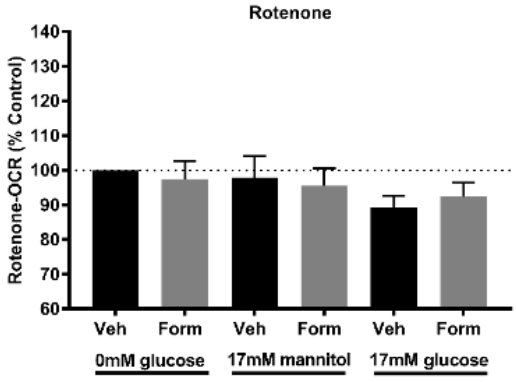
A


FCCP
FCCP-OCR (% Control)
140
130
120
110
100
90
80
70
60
α
β
Veh
Form
Veh
Form
Veh
Form
0mM glucose
17mM mannitol
17mM glucose

B


Antimycin A
AA-OCR (% Control)
140
130
120
110
100
90
80
70
60
α
β
Veh
Form
Veh
Form
Veh
Form
0mM glucose
17mM mannitol
17mM glucose

C


Rotenone
Rotenone-OCR (% Control)
140
130
120
110
100
90
80
70
60
Veh
Form
Veh
Form
Veh
Form
0mM glucose
17mM mannitol
17mM glucose

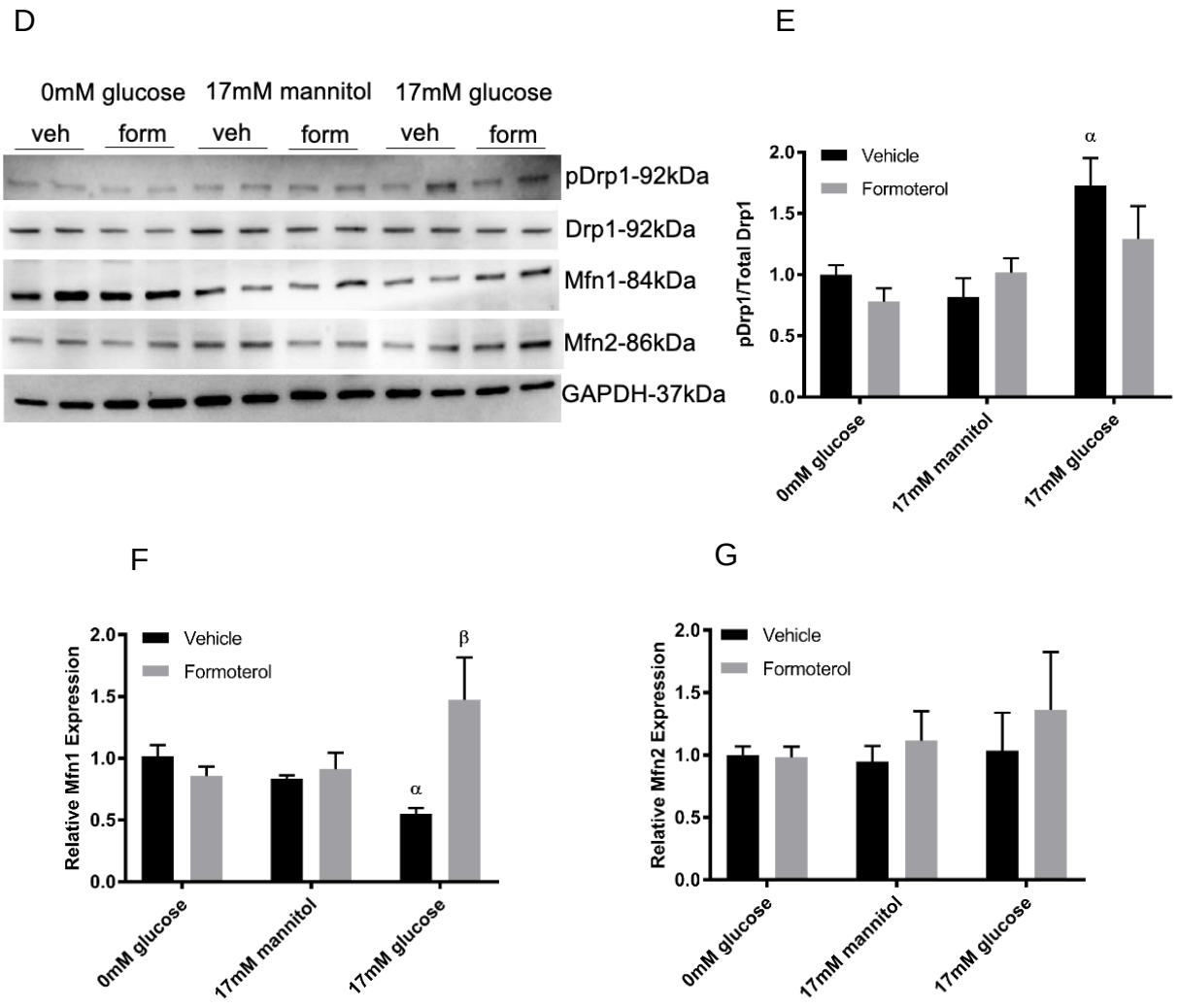


Figure 2.2. **Formoterol improves mitochondrial function and dynamics proteins in glucose-treated RPTC**. RPTC were grown in 0mM glucose, 17mM mannitol or 17mM glucose and co-treated with formoterol (30nM) for 96 hr. Seahorse XF96e Analyzer was used to measure (A) FCCP-OCR, (B) ETC complex III (antimycin A), (C) complex I (rotenone) function and (D) immunoblot and densitometry analysis of (E) pDrp1 S637, total Drp1, (F) Mfn1 and (G) Mfn2 expression. Data represented as mean±SEM, n=6-8. Statistical significance was determined by two-way ANOVA and Tukey-Kramer post-hoc

test. α=P<0.05 compared to 0mM glucose vehicle, β=P<0.05 compared to 17mM glucose vehicle.

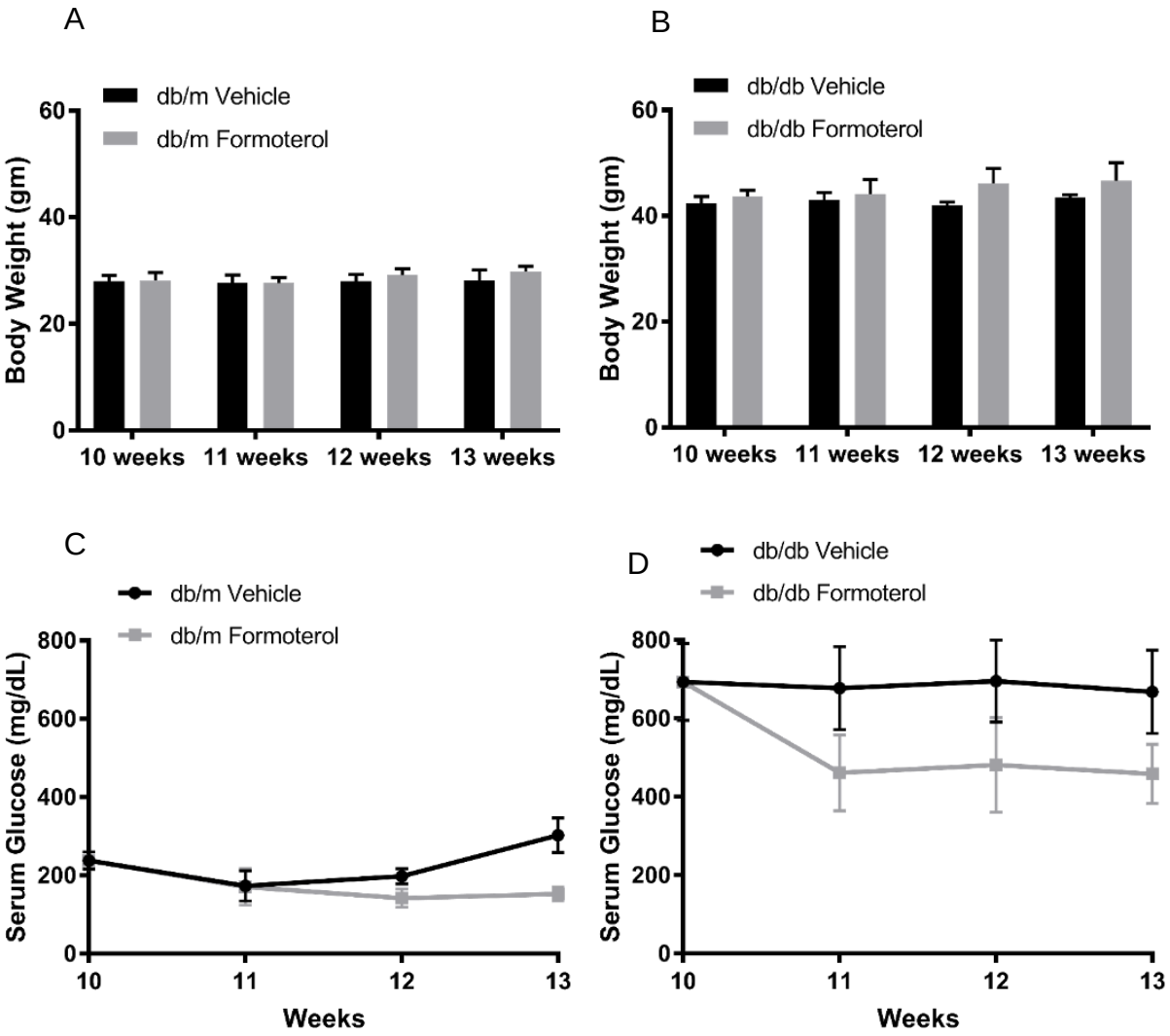
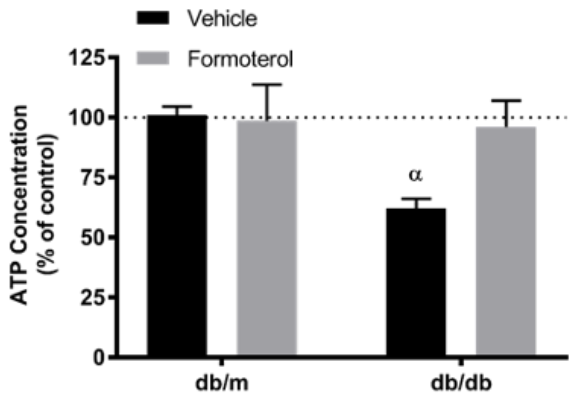
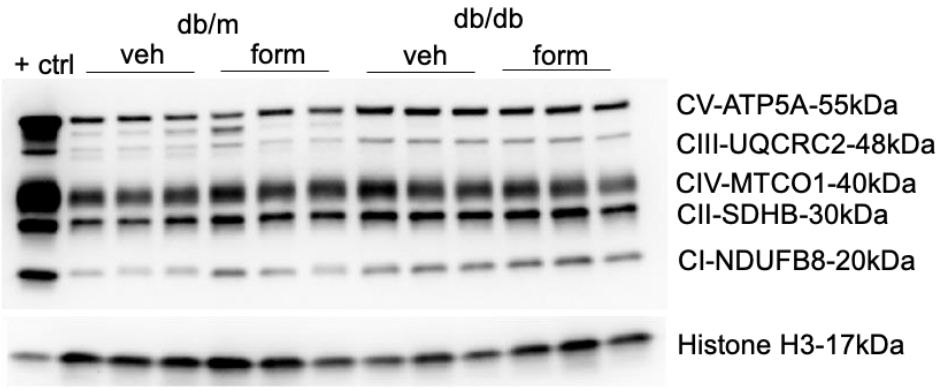


Figure 2.3. Formoterol has no effect on body weight and reduces serum glucose levels. Diabetic db/db and db/m control mice 10 weeks of age were treated with either vehicle (0.1% DMSO) or formoterol (1mg/kg) daily for 3 weeks. (A and B) body weight and (C and D) blood glucose levels from nondiabetic db/m and diabetic db/db mice between 8 and 13 weeks. Statistical significance was determined using one-way ANOVA and Tukey-Kramer post-hoc test. α=P<0.05 compared to db/m vehicle, β=P<0.05 compared to db/db vehicle. Data represented as mean±SEM, n=6.

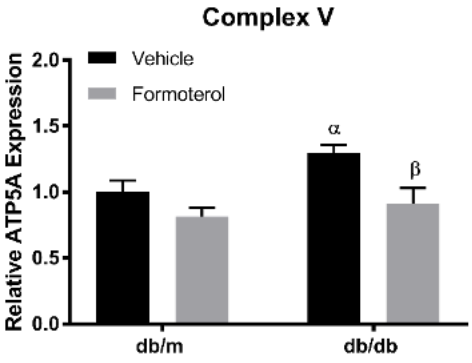
A


Vehicle
Formoterol
ATP Concentration (% of control)
125
100
75
50
25
0
α
db/m
db/db

B


db/m
db/db
+ ctrl
veh
form
veh
form
CV-ATP5A-55kDa
CIII-UQCRC2-48kDa
CIV-MTCO1-40kDa
CII-SDHB-30kDa
CI-NDUFB8-20kDa
Histone H3-17kDa

C


Complex V
Vehicle
Formoterol
Relative ATP5A Expression
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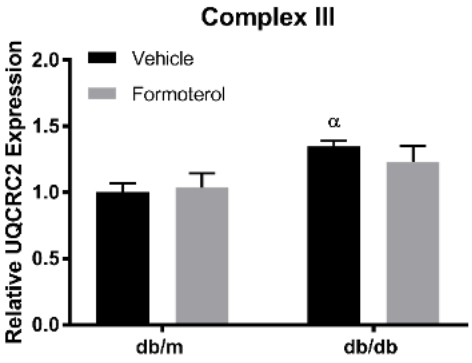
1.5
1.0
0.5
0.0
α
β
db/m
db/db

D


Complex III
Vehicle
Formoterol
Relative UQCRC2 Expression
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1.5
1.0
0.5
0.0
α
db/m
db/db

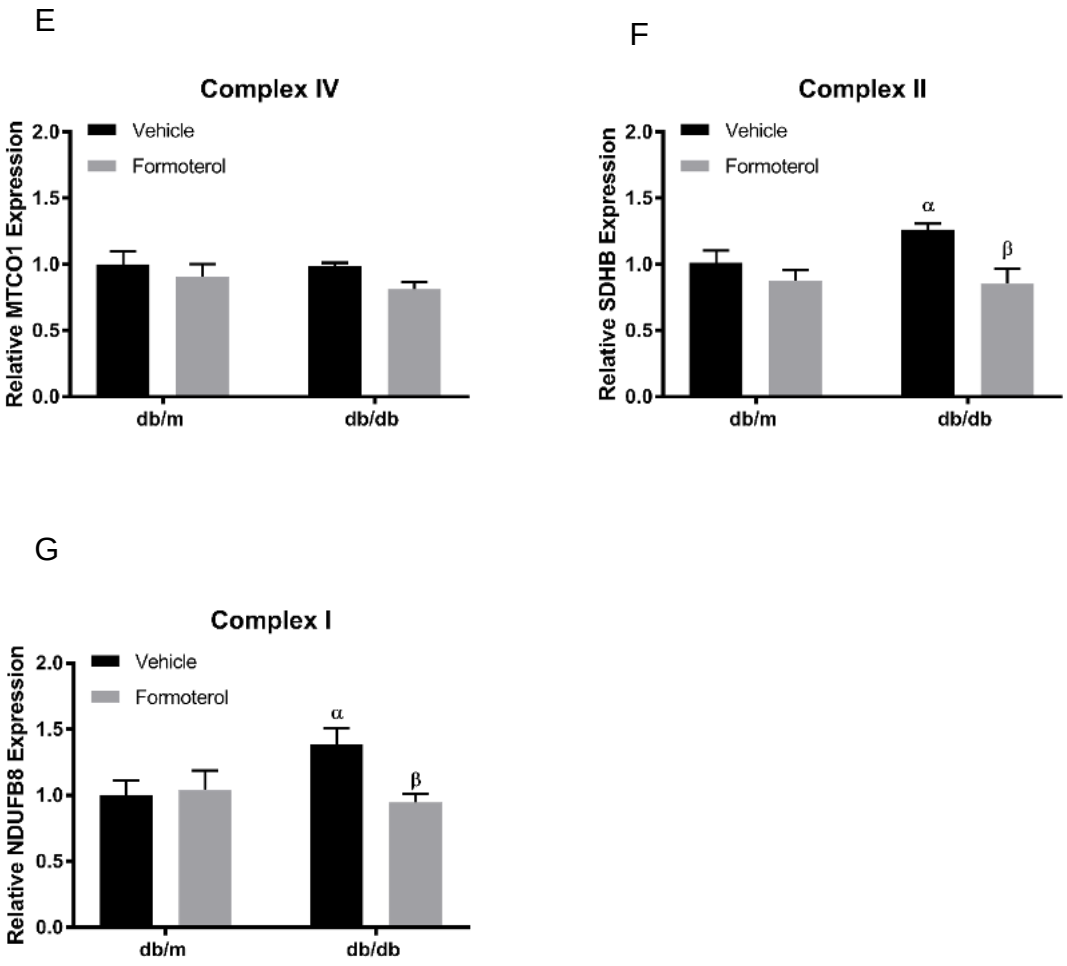


Figure 2.4. **Glucose decreases ATP content and alters ETC proteins in db/db mice.** Diabetic db/db and db/m control mice 10 weeks of age were treated with either vehicle (0.1% DMSO) or formoterol (1 mg/kg) daily for 3 weeks. Kidneys were harvested and renal cortical tissue was analyzed for (A) ATP content, (B) immunoblot for total oxphos proteins and densitometry analysis of (C) ATP5A, (D) UQCRC2, (E) MTCO1, (F) SDHB and (G) NDUFB8. Data represented as mean±SEM, n=6. Statistical significance was determined using two-way ANOVA and Tukey-Kramer post-hoc test. α=P<0.05 compared to db/m vehicle.

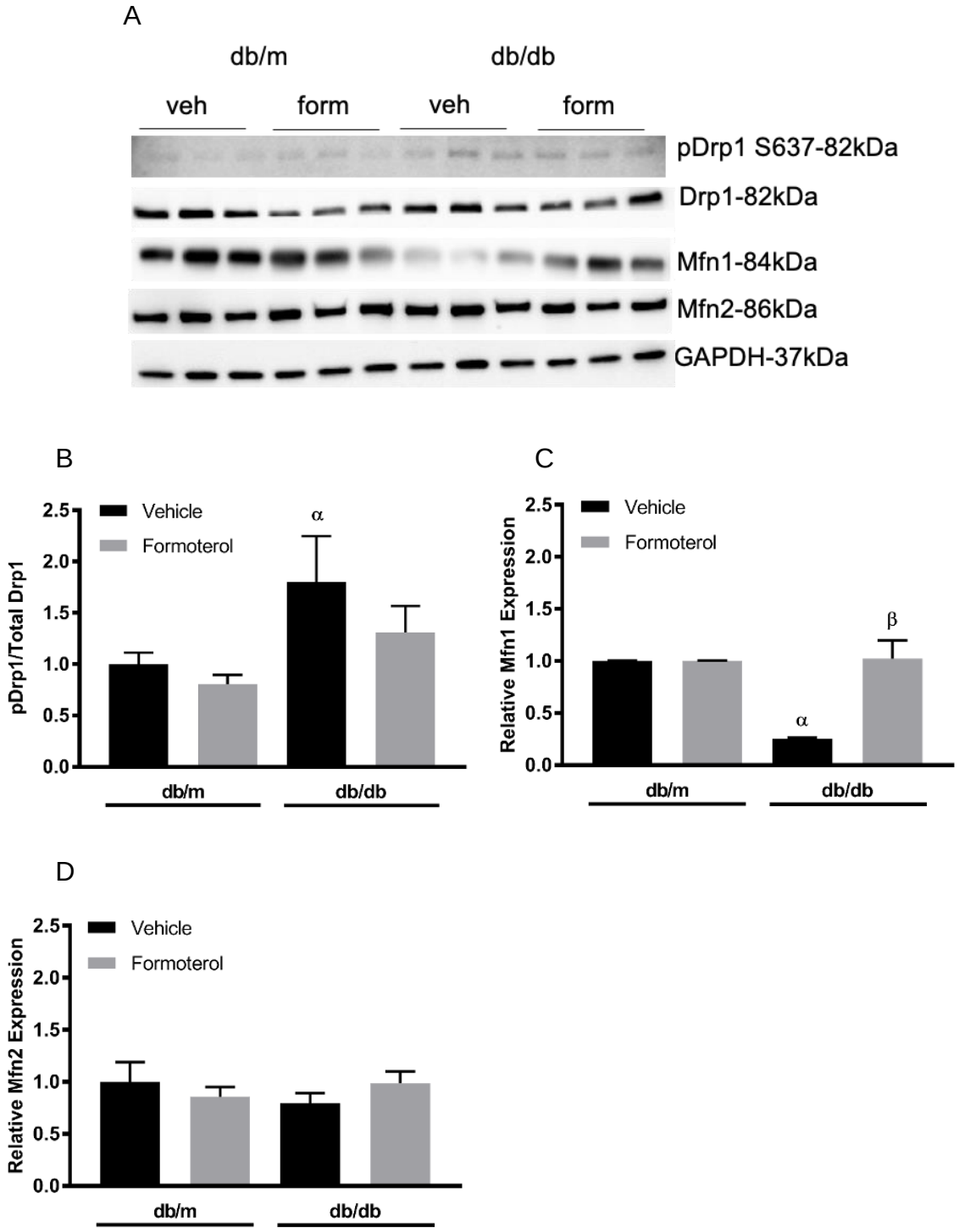


Figure 2.5. **Formoterol restores glucose-altered mitochondrial fission/fusion proteins in db/db mice**. Diabetic db/db and db/m control mice 10 weeks of age were treated with either vehicle (0.1% DMSO) or formoterol (1mg/kg) daily for 3 weeks. Kidneys

were harvested and protein was extracted from renal cortical tissue. (A) Immunoblot and densitometry analysis of (B) pDrp1 S637, total Drp1, (C) Mfn1 and (D) Mfn2. Statistical significance was determined using one-way ANOVA and Tukey-Kramer post-hoc test. α=P<0.05 compared to db/m vehicle, β=P<0.05 compared to db/db vehicle. Data represented as mean±SEM, n=6.

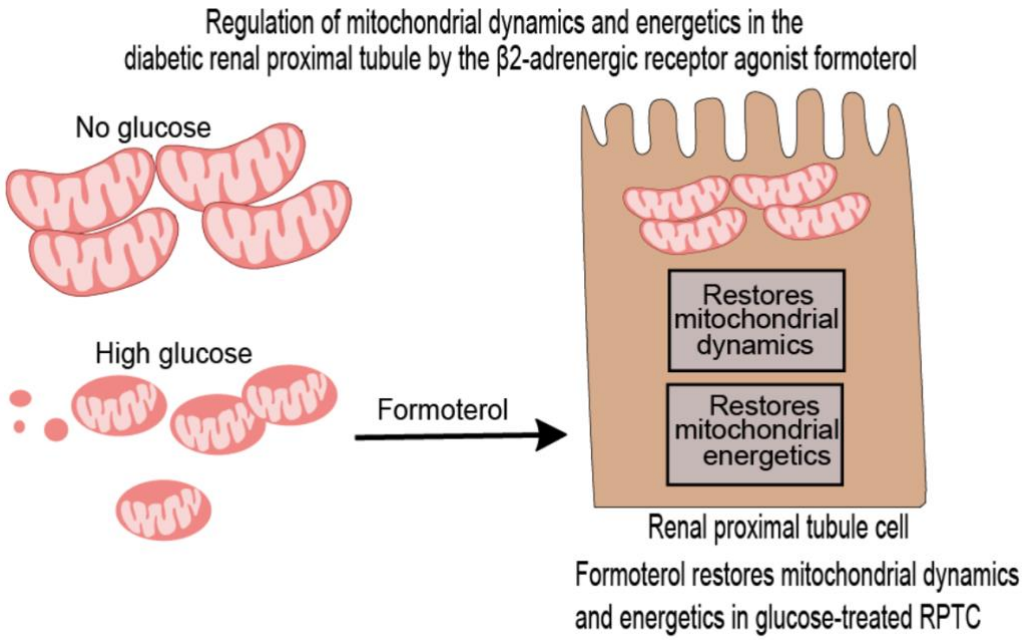


Figure 2.6. Regulation of mitochondrial dynamics and energetics in RPTC by formoterol. High glucose induces a pro-fission mitochondrial state in RPTC that is restored by formoterol.

Chapter 3:

The β2-adrenergic receptor agonist formoterol restores mitochondrial homeostasis in high glucose-induced renal proximal tubule cell injury through separate integrated pathways

3.1 Abstract

Mitochondrial dysfunction is a driving factor in the development and progression of diabetic kidney disease (DKD). Our laboratory discovered that the β_2-adrenoceptor agonist formoterol regulates mitochondrial dynamics in the hyperglycemic renal proximal tubule. Here, we identified signaling mechanisms through which formoterol regulates the mitochondrial fission protein Drp1 and the mitochondrial fusion protein Mfn1. Using primary cultures of renal proximal tubule cells (RPTC) exposed to high glucose, we investigated the role of formoterol on RhoA/ROCK1/Drp1 and Raf/MEK1/2/ERK1/2/Mfn1 signaling pathways using pharmacological inhibitors, and the effect of formoterol on these pathways. In the presence of high glucose, RhoA became hyperactive, leading to ROCK1-induced activation of Drp1. Using pharmacological inhibitors, we showed that formoterol signals through $G\beta\gamma$ of the β_2-adrenoceptor to decrease RhoA/ROCK1-mediated activation of Drp1. Inhibition of RhoA/ROCK1/Drp1 also restored maximal mitochondrial respiration. Formoterol restores this pathway by preventing the interaction of RhoA with the guanine nucleotide exchange factor p114RhoGEF. Formoterol also restores mitochondrial fusion through a separate $G\beta\gamma$-dependent mechanism composed of Raf/MEK1/2/ERK1/2/Mfn1. RPTC exposed to high glucose exhibited decreased Mfn1 activation, which was restored with formoterol. This effect was blocked after inhibition of

Gβγ. Pharmacological inhibition of Raf and MEK1/2 also restored Mfn1 activity. We demonstrate that glucose promotes the interaction between RhoA and p114RhoGEF, leading to increased RhoA/ROCK1/Drp1, and glucose decreases Mfn1 activity through activation of Raf/MEK1/2/ERK1/2. Formoterol restores these pathways and mitochondrial function in response to elevated glucose. Formoterol activates three separate pathways that promote mitochondrial biogenesis, decreased fission and increased fusion in RPTC, further supporting its potential as a therapeutic for DKD.

3.2 Introduction

Diabetic kidney disease (DKD) is a prevalent metabolic disease and is the most common cause of end stage renal disease (ESRD) (124). Despite existing therapies that target hypertension and hyperglycemia, these treatments only slow the progression to ESRD. Thus, there is a need for the identification and development of new pharmacological therapeutics to treat DKD.

In type 2 diabetes, early hyperglycemia and glomerular hyperfiltration affect renal glomerular and proximal tubular function. Hyperglycemia exposes the proximal tubule to increased amounts of filtered glucose, which in turn leads to increased glucose reabsorption (4). The increased tubular glucose load results in a number of pathophysiological changes: proximal tubule growth, upregulation of sodium-glucose cotransporter 2 (SGLT2), inflammation, mitochondrial dysfunction and eventually leading to tubulointerstitial fibrosis (19, 41, 66, 141-143). Although there are clear and distinct

changes in proximal tubular function in DKD, the mechanisms underlying these changes are understudied.

The kidney is a highly metabolic organ that relies heavily on mitochondrial oxygen consumption to account for the energy requirements of tubular reabsorption (8, 9). Mitochondrial dysfunction has been identified as a key event in the early stages of hyperglycemia leading to disease progression. Renal mitochondrial dysfunction encompasses multiple functional changes. Studies have shown that expression and activity of the master regulator of mitochondrial biogenesis (MB) peroxisome proliferator-activated receptor gamma co-activator 1 alpha (PGC1α) is downregulated in proximal tubules of diabetic animals, leading to disease progression (25).

In addition, studies report that mitochondrial dynamics is altered in diabetic db/db mice and in glomerular podocytes exposed to high glucose (2, 3). These changes in mitochondrial dynamics were mediated by the mitochondrial fission protein dynamin-like protein 1 (Drp1). When Drp1 was knocked down or pharmacologically inhibited, mitochondrial function was restored. Importantly, restoration of mitochondrial dynamics and function further led to significant improvement of hallmark features of DKD including glomerular scarring, albuminuria and mesangial matrix expansion. It has also been reported that Mfn1 expression decreases in response to high glucose, although the extent of knowledge regarding Mfn1 in DKD is limited (20). These studies provide evidence that mitochondrial function is altered in response to glucose and improving aspects of mitochondrial dysfunction such as mitochondrial dynamics can be an effective therapeutic strategy in DKD.

We previously demonstrated that in RPTC exposed to high glucose, phosphorylation of the mitochondrial fission protein Drp1 was increased and expression the mitochondrial fusion protein Mfn1 was decreased, indicating that there is an imbalance in RPTC mitochondrial dynamics in the presence of glucose (19). Interestingly, the same effects were observed in renal cortical tissue of early diabetic db/db mice. Despite these clear and distinct alterations in mitochondrial dynamics proteins, the signaling mechanisms underlying these effects remain unknown. Studies have identified that Drp1 phosphorylation in hyperglycemia is mediated by Rho-associated protein kinase 1 (ROCK1) (17). Furthermore, separate studies show that Ras homolog family member A (RhoA) is responsible for ROCK1 activation and subsequent Drp1 phosphorylation (144). Little is known about the role of Mfn1 and its associated signaling pathways in high glucose. However, it has been shown that MEK1/2/ERK1/2 regulates Mfn1 function (145). In addition, it has been demonstrated that Raf, the upstream activator of MEK1/2 is also upregulated in the presence of glucose (146).

Our previous study showed that altered Drp1 phosphorylation and Mfn1 expression in diabetic db/db mice as well as in RPTC exposed to high glucose was restored by treatment with the β_2-adrenoceptor agonist formoterol. However, the mechanisms as to how formoterol activation of the β_2-adrenoceptor regulates mitochondrial dynamics have yet to be determined. In separate studies, we also demonstrated that formoterol promotes recovery from acute kidney injury (AKI) by stimulating MB (136, 137). Despite the well-known classical pathway of β_2-adrenoceptor activation, defined by Gαs stimulation of adenylyl cyclase (AC) and cAMP production, we discovered that formoterol signals through G$\beta\gamma$ to activate Akt/eNOS/sGC/PGC1α to

induce MB (121). Based on these findings, our hypothesis was formoterol signals through the Gβγ subunit of the β_2-adrenoceptor to regulate RhoA/ROCK1/Drp1 and Raf/MEK1/2/ERK1/2/Mfn1 to restore the imbalance between mitochondrial fission and fusion in DKD.

3.3 Methods

3.3.1 Material

Carvedilol (#2685), gallein (#3090),Y-27632 (#1254), Mdivi-1 (#398250) were purchased from Tocris Bioscience (Bristol, UK). PLX4032 (S1267) and GSK11201 (S2673) were purchased from Selleckchem. Formoterol (F9552) and CCG-1423 (555558) were purchased from Sigma-Millipore.

3.3.2 In vitro studies

Female New Zealand White rabbits (1.8-2 kg) were purchased from Charles River (Oakwood, MI/Canada). RPTC were isolated using the iron oxide perfusion method and grown in 35-mm tissue culture dishes under improved culture conditions similar to what is observed *in vivo (Nowak, 1995, Improved culture conditions stimulate gluconeogenesis in primary cultures of renal proximal tubule cells).* The culture medium was a 1:1 mixture of Dulbecco's modified Eagles medium /F-12 (without glucose, phenol red or sodium pyruvate) supplemented with 15mM HEPES buffer, 2.5mM L-glutamine, 1μM pyroxidine HCl, 15mM sodium bicarbonate and 6 mM lactate. Hydrocortisone (50nM), selenium (5ng/ml), human transferrin (5μg/ml), bovine insulin (10nM) and L-ascorbic acid-2-phosphate (50μM) were added to fresh culture medium. Cells grown in the presence of

glucose or mannitol were supplemented with 17mM D-glucose or 17mM D-mannitol (osmotic control). Confluent RPTC were used for all experiments.

3.3.3 GTP pulldown assay

GTP agarose beads (30μl) purchased from Abcam (Cambridge, MA) were washed three times with immunoprecipitation (IP) buffer (25mM Tris, 150mM NaCl) and incubated with 200μg protein overnight at 4^{o}C with rotation. The following day, the bead-protein mixture was washed three times with IP buffer (25mM Tris Base, 150mM NaCl). Beads were boiled at 95^{o}C with laemmli sample buffer for 5 min and the samples were centrifuged to harvest the pulldown proteins. GTP-bound proteins and sample input proteins were resolved on 4-15% SDS-PAGE gels, transferred onto nitrocellulose membranes and blotted for either Drp1, RhoA or Mfn1.

3.3.4 Immunoprecipitation

Dynabeads Protein G Immunoprecipitation kit (ThermoFisher, Waltham, MA) was used to immunoprecipitate RhoA. Proteins (200μg) were incubated with Pierce Protein A/G agarose beads (ThermoFisher) for 2 hr and centrifuged at 14,000g for 10 min at 4^{o}C. Dynabeads were incubated with RhoA antibody (1:100) for 4 hr at room temperature. Supernatants from precleared lysates were added to the Dynabeads-RhoA antibody complex and incubated overnight at 4^{o}C with rotation. RhoA was immunoprecipitated and eluted (denaturing elution) based on the manufacturer's instructions. The resulting supernatant was loaded onto 4-15% SDS-PAGE gels with a 5% input control, transferred onto nitrocellulose membranes and blotted for p114RhoGEF. Membranes were incubated and visualized as described below.

3.3.5 Immunoblot analysis

Protein was extracted from RPTC cultures using RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, pH 7.4). Protease inhibitor cocktail (1:100), 1mM sodium fluoride and 1mM sodium orthovanadate (Sigma-Aldrich, St. Louis, MO) were added fresh before each extraction. Equal protein quantities (10μg) were loaded onto 4-15% SDS-PAGE gels, resolved by gel electrophoresis and transferred onto nitrocellulose membranes (Bio-Rad). Membranes were blocked with either 5% nonfat milk or 5% BSA in TBST and incubated overnight with primary antibody at 4°C with agitation. Primary antibodies used in these studies include Drp1 (1:1000) (#5991), RhoA (1:1000) (#2117) used for IP, and GAPDH (1:1000) (#5174) were all purchased from Cell Signaling Technologies (Danvers, MA). Mfn1 (1:1000) (ab#2211661), p114RhoGEF (ab#96520) and RhoA (1:1000) (ab#187027) were purchased from Abcam. Membranes were incubated with the appropriate horseradish peroxidase conjugated secondary antibody before visualization using enhanced chemiluminescence (Thermo Scientific, Waltham, MA) and the GE ImageQuant LAS4000 (GE Life Sciences, Marlborough, MA). Optical density was determined using Bio-Rad Image Lab 6.0.

3.3.6 Analysis of oxygen consumption

Cultured RPTC were passaged onto 96-well XF96 extracellular flux analyzer plates (Agilent Technologies, Santa Clara, CA) at a cell density of $1.6x10^4$ cells per well and grown in media containing 0mM glucose, 17mM mannitol or 17mM glucose for 96 hr. Cells were treated with pharmacological inhibitors at 72 hr after plating, for a period of 24

hr. Basal OCR was measured three times using the Seahorse Bioscience XF96 Analyzer before injection of carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) (2μM) (Sigma Aldrich) to measure OCR as previously described (Beeson et al., 2010). OCR was reported as picomoles per minute, and the results were normalized as a percentage of vehicle control (DMSO).

3.3.7 Statistical analysis

All data are shown as mean±SEM. Two-way analysis of variance followed by Tukey's post hoc test was performed for comparisons of multiple groups. $P<0.05$ was considered statistically significant. All statistical tests were performed using the GraphPad Prism software (GraphPad Software, San Diego, CA).

3.4 Results

3.4.1 Effect of high glucose on RhoA and Drp1 activity

We previously demonstrated that phosphorylation of Drp1 was increased at Ser637 in the presence of high glucose and was decreased upon formoterol treatment. To determine whether this increase in phosphorylation also led to increased Drp1 activity and to determine the involvement of RhoA, a GTP-pulldown assay to determine Drp1 and RhoA activity. RPTC exposed to high glucose had increased GTP-bound RhoA (Fig 3.1A) and Drp1 (Fig 3.11B) compared to 0mM glucose and 17mM mannitol controls. Formoterol treatment reduced both RhoA and Drp1 activity to control levels.

To determine the involvement of the RhoA/ROCK1/Drp1 signaling pathway, CCG-1423, a pharmacological inhibitor of RhoA was used. In RPTC exposed to high glucose,

RhoA activity increased in the presence of glucose. Treatment with either formoterol or CCG-1423 prevented the increase in RhoA activity (Fig 3.2A). In addition, treatment with CCG-1423 prevented glucose-induced activity of Drp1 (Fig 3.2B). Seahorse XF96 analysis was used to determine the effect of RhoA inhibition on FCCP-OCR, a marker of MB. Glucose exposure did not change basal respiration in any treatment group (Fig 3.2D and F). However, after FCCP injection, maximal respiration decreased in RPTC grown in the presence of high glucose compared to controls, and RhoA inhibition restored maximal respiration (Fig 3.2E and G).

3.4.2 Effect of ROCK1 inhibition on Drp1 activity and mitochondrial function

To determine the involvement of ROCK1, a downstream kinase of RhoA, we treated RPTC with the ROCK1 inhibitor Y-27632 and measured Drp1 activity. RPTC treated with either formoterol or Y-27632 restored Drp1 activity to control levels (Fig 3.2C). Similarly to what was observed after RhoA inhibition, inhibition of ROCK1 had no effect on basal-OCR under any conditions (Fig 3.2H and J) and restored maximal mitochondrial respiration to the same level as controls (Fig 3.2I and K). The specific Drp1 inhibitor Mdivi-1 was used to determine whether the effect of glucose on mitochondrial respiration and MB was a result of altered mitochondrial fission. Glucose did not change basal respiration, nor did treatment with formoterol or Mdivi-1 (Fig 3.3A). Formoterol increased FCCP-OCR in 0mM glucose and mannitol controls compared to vehicle, whereas Mdivi-1 did not (Fig 3.3B). However, RPTC grown in high glucose, both formoterol and Mdivi-1 treatment restored FCCP-OCR.

3.4.3 Effect of formoterol activation of the β_2-adrenoceptor on RhoA signaling

To determine if modulation of the RhoA/ROCK1/Drp1 signaling pathway was mediated through the β_2-adrenoceptor, carvedilol, a β_2-adrenoceptor antagonist with a high affinity for the β_2-adrenoceptor (K_D=-9.40±0.08) to block the effect of formoterol on the receptor. While high glucose increased RhoA and Drp1 activity and formoterol restored both RhoA and Drp1 activity, carvedilol blocked this effect (Fig 3.4A and B). We previously discovered that formoterol activates the G$\beta\gamma$ subunit of the β_2-adrenoceptor to induce MB. Therefore, to determine whether formoterol works through G$\beta\gamma$ signaling to regulate Drp1. RPTC were grown in the presence or absence of glucose with the G$\beta\gamma$ inhibitor gallein. Gallein blocked the ability of formoterol to restore both RhoA and Drp1 activity (Fig 3.5A and B). Because RhoA is active when bound to GTP and this process is regulated by GDP-GTP exchange via RhoGEFs, RhoA was immunoprecipitated to determine potential interactions with RhoGEF proteins that might be regulating RhoA activity. Among these RhoGEFs, we found that the interaction between RhoA and p114RhoGEF was increased in the presence of glucose (Fig 3.6). Treatment with formoterol reduced the interaction between RhoA and p114RhoGEF to control levels. Interestingly, co-treatment with gallein blocked the ability of formoterol to prevent this interaction.

3.4.4 Effect of high glucose and formoterol on Mfn1 signaling

We previously discovered that in addition to alteration of mitochondrial fission via Drp1, expression of the mitochondrial fusion protein Mfn1 was decreased in models of DKD. To investigate the signaling mechanism responsible for altered Mfn1 expression,

RPTC were treated with the Raf inhibitor PLX4032. Since Mfn1 is also a GTPase and its activity can be detected by measuring GTP-bound protein, we used the same GTP pulldown assay to measure Mfn1 activity. In RPTC treated with vehicle in the presence of high glucose, Mfn1 activity was decreased compared to 0mM glucose and mannitol controls (Fig 3.7A). Cells treated with either formoterol or PLX4032 restored Mfn1 activity to control levels. Since Raf phosphorylates MEK1/2, the MEK1/2 inhibitor GSK11202 was used to determine the role of MEK1/2 and ERK1/2 in Mfn1 activation. Treatment with GSK11202 blocked ERK1/2 phosphorylation in RPTC grown in 0mM glucose, 17mM mannitol and 17mM glucose, while treatment with either vehicle or formoterol had no effect (Fig 3.7B). In addition, both GSK11202 and formoterol treatment prevented the decrease in Mfn1 activity compared to 0mM glucose and 17mM mannitol controls (Fig 3.7C).

To determine whether the Raf/MEK1/2/ERK1/2/Mfn1 pathway is also regulated by formoterol activation of Gβγ, we used gallein to block Gβγ signaling and subsequently measured Mfn1 activity. Formoterol, gallein or co-treatment of formoterol+gallein had no statistical effect on GTP-bound Mfn1 in either 0mM glucose or 17mM mannitol control groups (Fig 3.8). In the presence of glucose, GTP-Mfn1 was significantly reduced compared to controls. While formoterol prevented the decrease in Mfn1 activity, co-treatment with gallein blocked this effect. Importantly, phosphorylation of Akt after formoterol or gallein treatment was unchanged in all groups (Fig. 3.9).

3.5 Discussion

This study investigated the signaling pathways associated with altered mitochondrial dynamics in RPTC exposed to high glucose. Increased expression and activation of Drp1 has been implicated in multiple cell types along the nephron and contributes to mitochondrial dysfunction in models of DKD and RPTC (2, 3, 19, 147). Prior studies have identified that ROCK1 is an activator of Drp1 (17). Separate studies have identified that RhoA also leads to increased Drp1 phosphorylation and translocation to the mitochondria in a ROCK1-dependent manner (144). Aside from RhoA/ROCK1, Drp1 is also phosphorylated by protein kinase D (PKD) at Ser637 to initiate mitochondrial fission (148). In addition, Drp1 is also activated by protein kinase A (PKA)-induced phosphorylation of Ser637, although phosphorylation by PKA has been demonstrated to have an inhibitory effect on Drp1 (16). However, this study did not assess the effects of these upstream kinases on Drp1 activity in response to glucose.

The RhoA/ROCK1/Drp1 pathway is hyperactivated in RPTC treated with high glucose. Using inhibitors of these proteins, we confirmed this signaling pathway and determined that formoterol acts through the $G\beta\gamma$ subunit of the β_2-adrenoceptor to inhibit p114RhoGEF interaction with RhoA. By preventing this interaction, there was a restoration of both RhoA and Drp1 activity. Despite this finding, it is still unclear exactly how $G\beta\gamma$ blocks the interaction between p114RhoGEF and RhoA. Using pharmacological inhibitors targeting RhoA, ROCK1 and Drp1, maximal mitochondrial respiration was restored, indicating that in addition to its role in mitochondrial fission, Drp1 also plays a role in regulating mitochondrial respiration.

Prior work has noted that components of the proposed Mfn1 pathway, such as Raf, MEK1/2 and ERK1/2 are upregulated in response to elevated glucose (146, 149, 150). Despite this knowledge, this signaling pathway has not yet been elucidated in renal cell types exposed to hyperglycemic conditions. One study by Pyakurel et al. demonstrated that MEK1/2/ERK1/2 phosphorylates Mfn1 to modulate its activity as a mechanism of apoptosis regulation (145). Based on these studies, there is evidence linking MEK1/2/ERK1/2 to Mfn1 activation. In support of these findings, this study demonstrates that by using pharmacological inhibitors of Raf and MEK1/2, Mfn1 activity is restored. Importantly, when Gβγ was blocked with gallein, formoterol lost its effect on Mfn1, indicating that this mitochondrial fusion pathway is restored by activating Gβγ. Our previous study showed that formoterol induces MB through Gβγ-induced phosphorylation of Akt. However, this study demonstrates that in RPTC, neither glucose nor formoterol effects the phosphorylation status of Akt, indicating that the pathways through which formoterol restores mitochondrial fission/fusion are separate from that which regulates MB. It is important to note that the present study does not determine exactly how formoterol activation of Gβγ directly leads to decreased Raf activation to ultimately restore Mfn1. However, it has been previously reported that both Gα and Gβγ can regulate MAPK signaling pathways (151, 152), further indicating that the resulting outcome of G-protein subunit activation likely relies on context and stimulus specificity.

MB plays an important role maintaining mitochondrial homeostasis and in regulating cellular metabolism. Pharmacologically activating MB has shown potential as a therapeutic strategy (24). It has previously been determined that formoterol activation of Gβγ leads to PI3K-dependent activation of Akt/eNOS/sGC in RPTC (121), and

activation of this pathway leads to increased PGC1α and MB. The study by Cameron et al. determined that formoterol activation of Gβγ-dependent signaling is important for GPCR-mediated MB. In this study, we show that that in addition to its role in activating Akt/eNOS/sG to induce MB, formoterol also restores mitochondrial fission through p114/RhoA/ROCK1/Drp1 and mitochondrial fusion through Raf/MEK1/2/ERK1/2/Mfn1 through Gβγ-dependent mechanisms. Although formoterol induces three separate and distinct pathways, they are integrated and work simultaneously to restore mitochondrial homeostasis in RPTC in response to high glucose injury (Fig 3.10). It is widely known that β-arrestins (β-arr1) 1 and 2 are required for ERK1/2 activation via scaffolding of Raf/MEK1/2/ERK1/2. However, a study by O'Hayre et al. demonstrated that β-arr1 and β-arr2 are dispensable for β2-AR-dependent ERK activation (157). Rather, the authors demonstrated that β2-ARs signal through Gαs and Gβγ to activate the tyrosine kinase Src and the guanine nucleotide exchange factor SOS to activate Raf, MEK and ERK. These findings support our results which demonstrate that formoterol signals through Gβγ to restore mitochondrial homeostasis.

While current therapies for DKD are effective at targeting hypertension and hyperglycemia, there is no existing drug that improves disease progression. A common property of these therapeutics lies in their ability to modulate mitochondrial function. Studies evaluating the effects of angiotensin receptor type 1 and type 2 receptor (AT1R and AT2R) blockers showed that olmesartan restored altered expression of TCA cycle enzymes and the superoxide generating enzyme Nox2 (62). Another antagonist, losartan, provided renal mitochondrial protection from oxidative injury (63). Interestingly, studies evaluating the effects of anti-hyperglycemic agents on mitochondrial effects showed that

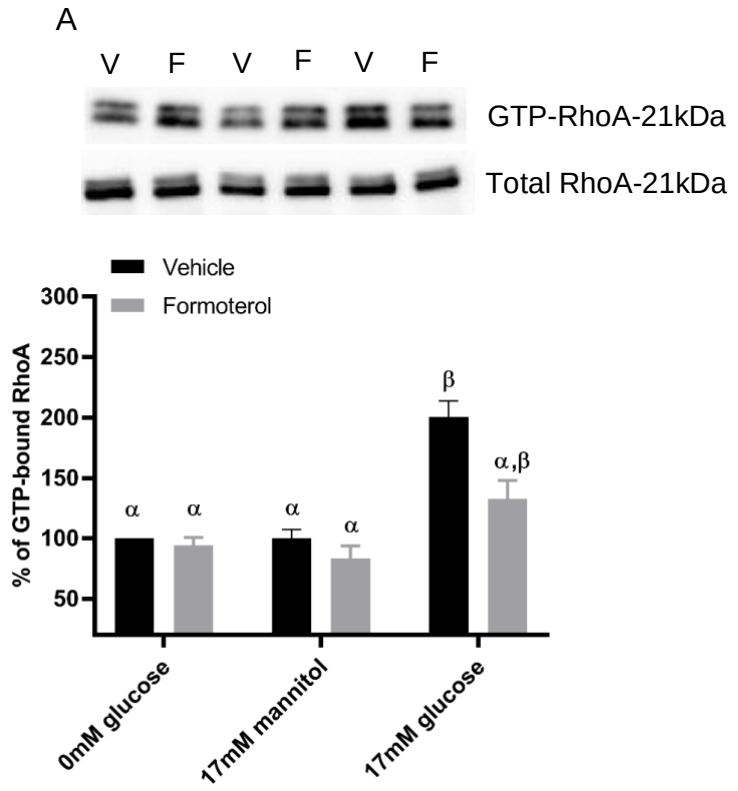
the SGLT2 inhibitor dapagliflozin also demonstrated a protective effect on mitochondrial function and stimulated PGC1α (72). These studies provide evidence that mitochondrial dysfunction is a driving factor in the development and progression of DKD and support the hypothesis that mitochondrial therapies can improve both renal function and hallmark features of DKD.

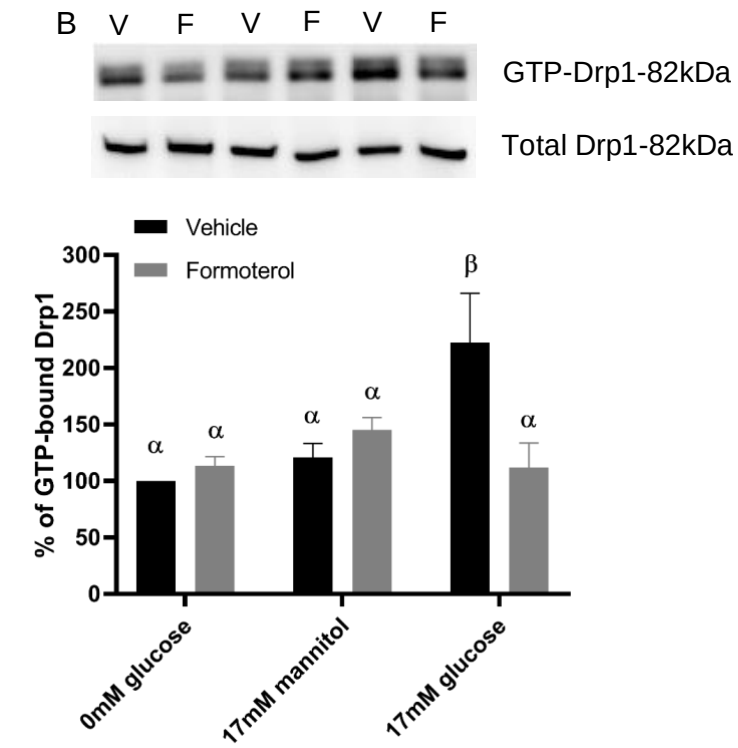
Formoterol has shown to be a promising therapeutic for the treatment of several kidney diseases. In addition to its beneficial effects on recovery from AKI, it has also been demonstrated that formoterol accelerates podocyte recovery from glomerular injury by inducing MB (122), providing evidence that improving mitochondrial function has a beneficial therapeutic effect on renal diseases. Since formoterol is already an FDA-approved drug for the treatment of asthma, its use as a repurposed therapeutic for DKD would be both a time and cost-effective strategy. Although it has been demonstrated that formoterol can restore mitochondrial function leading to improved kidney function, the mechanisms through which formoterol exerts these effects are still being uncovered. In this study, we show that formoterol signals through novel, distinct and separate yet integrated mechanistic pathways to restore mitochondrial homeostasis in RPTC.

3.6 Conclusions

As it has previously been established that proteins involved in mitochondrial dynamics are altered in hyperglycemia, this study elucidated the mechanisms through which glucose and formoterol regulate the activity of the mitochondrial fission/fusion proteins Drp1 and Mfn1 as depicted in Figure 3.9. Prior studies have identified the involvement of RhoA/ROCK1 in Drp1 activation, however this is the first study to report that the RhoA/ROCK1 pathway is hyperactive in RPTC exposed to high glucose, and importantly, the observed overactivation is a result of increased interaction between RhoA and the guanine nucleotide exchange factor p114RhoGEF. In addition, this is the first study to report that formoterol regulates the p114/RhoA interaction by activation $G\beta\gamma$ of the β2-AR. In addition, we are the first to show that Raf/MEK1/2/ERK1/2 regulates Mfn1 in RPTC exposed to high glucose, and this pathway is also regulated by formoterol.

3.7 Figures and Figure Legends





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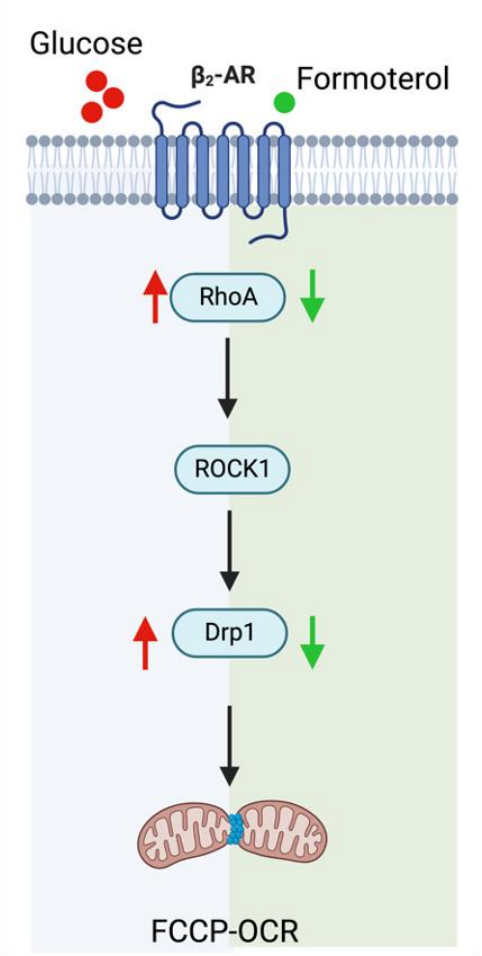
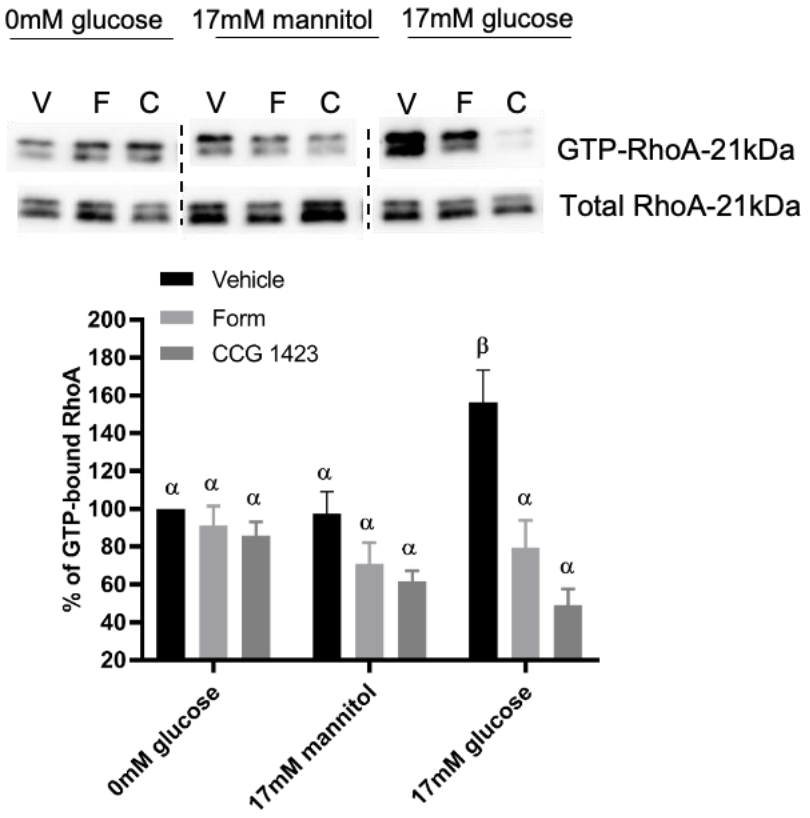
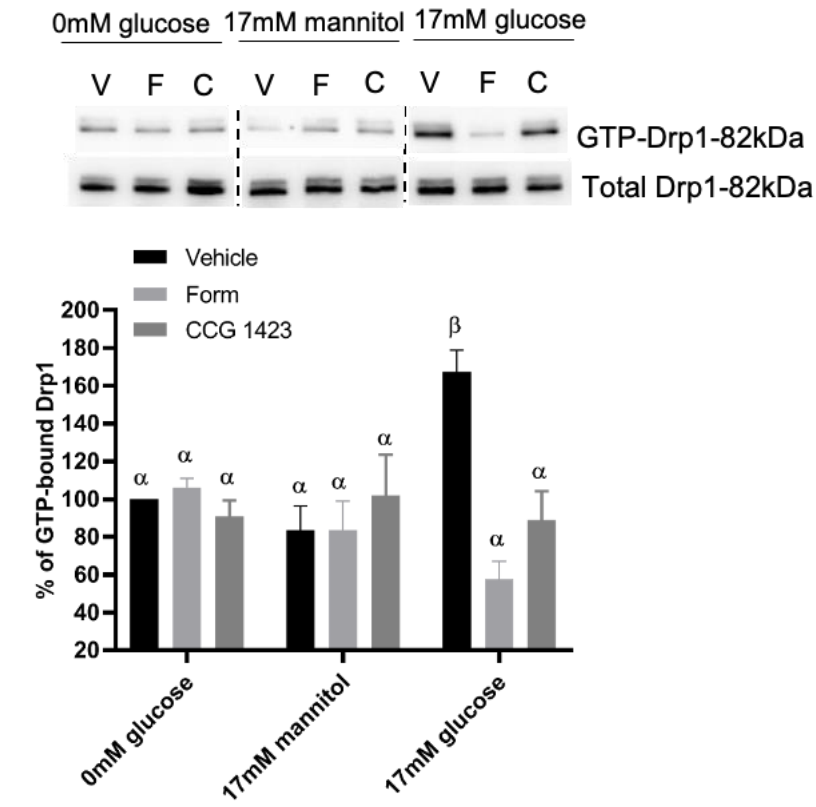


Figure 3.1. **Formoterol restores RhoA and Drp1 activity in glucose treated RPTC**. RPTC were grown in 0mM glucose, 17mM mannitol or 17mM glucose for 96 hr. At 72 hr, cells were treated with either vehicle (0.1% DMSO) or formoterol (30nM) for 24 hr. Cells were harvested and proteins were subjected to immunoblot analysis of (A) GTP-bound RhoA and total RhoA, and (B) GTP-bound Drp1 and total Drp1. Data represent mean±SEM, n=6. Statistical significance was determined by two-way ANOVA and Tukey-Kramer post-hoc test. Different Greek letters signify statistical differences.

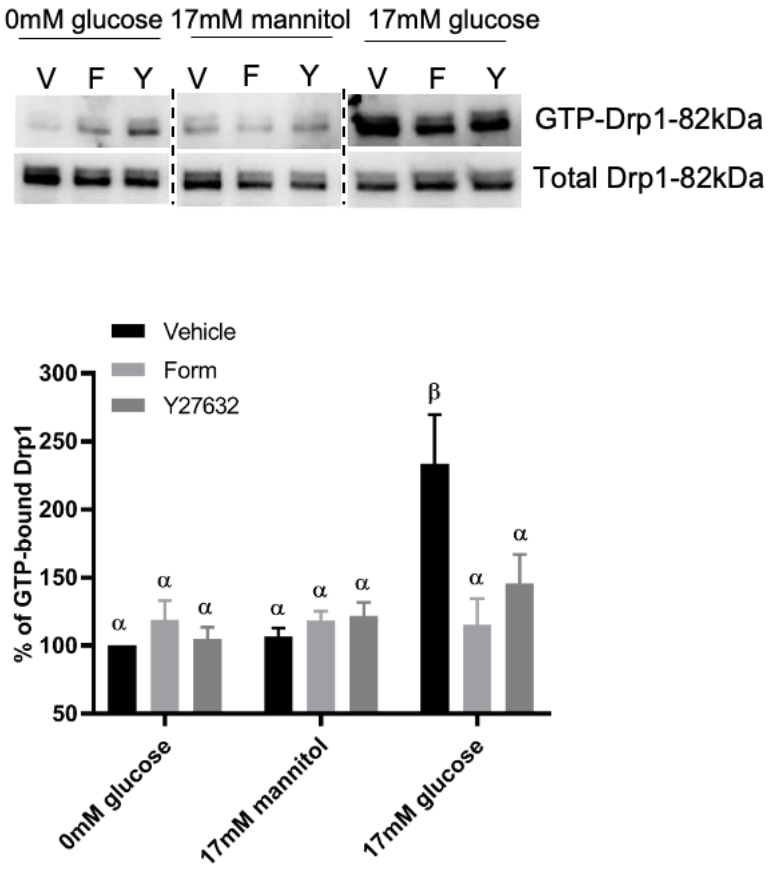
A


0mM glucose
17mM mannitol
17mM glucose
V F C V F C V F C
GTP-RhoA-21kDa
Total RhoA-21kDa
Vehicle
Form
CCG 1423
% of GTP-bound RhoA
200
180
160
140
120
100
80
60
40
20
α
β
0mM glucose
17mM mannitol
17mM glucose

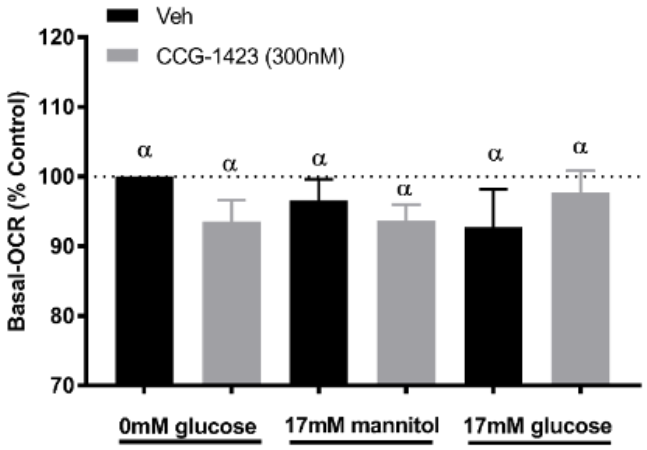
B


0mM glucose
17mM mannitol
17mM glucose
V F C V F C V F C
GTP-Drp1-82kDa
Total Drp1-82kDa
Vehicle
Form
CCG 1423
% of GTP-bound Drp1
200
180
160
140
120
100
80
60
40
20
α
β
0mM glucose
17mM mannitol
17mM glucose

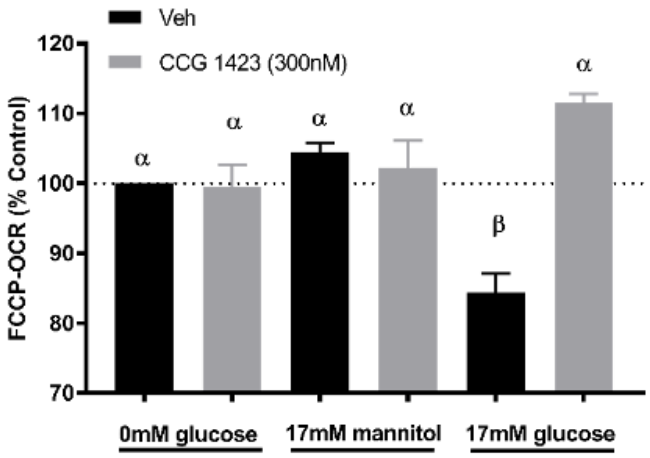
C


0mM glucose
17mM mannitol
17mM glucose
V F Y V F Y V F Y
GTP-Drp1-82kDa
Total Drp1-82kDa
Vehicle
Form
Y27632
% of GTP-bound Drp1
300
250
200
150
100
50
α
α
α
α
α
α
β
α
α
0mM glucose
17mM mannitol
17mM glucose

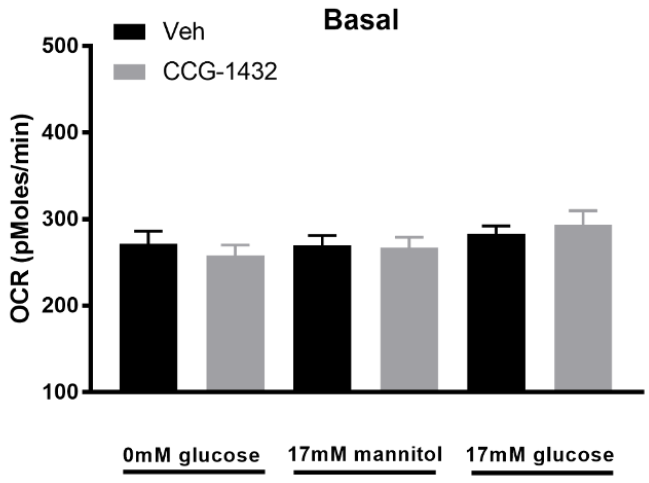
D


Veh
CCG-1423 (300nM)
Basal-OCR (% Control)
120
110
100
90
80
70
α
α
α
α
α
α
0mM glucose
17mM mannitol
17mM glucose

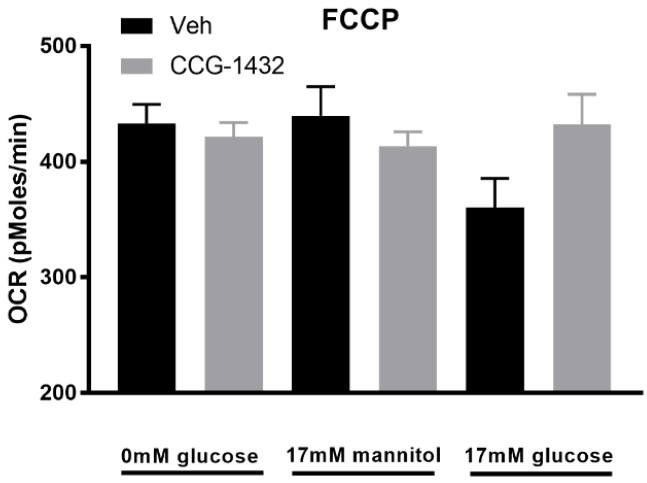
E


Veh
CCG 1423 (300nM)
FCCP-OCR (% Control)
120
110
100
90
80
70
α
α
α
α
β
α
0mM glucose
17mM mannitol
17mM glucose

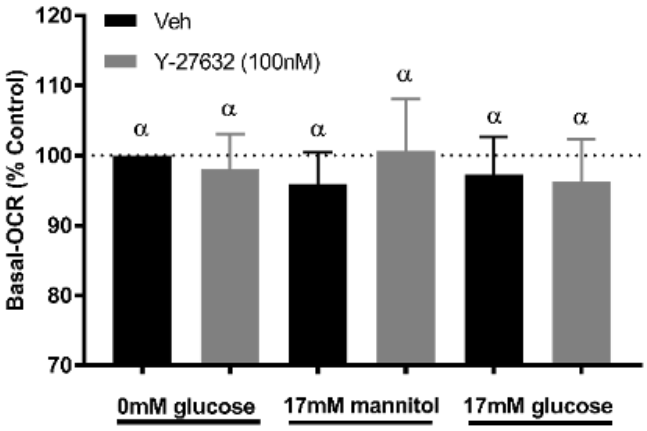
F


Basal
Veh
CCG-1432
OCR (pMoles/min)
500
400
300
200
100
0mM glucose
17mM mannitol
17mM glucose

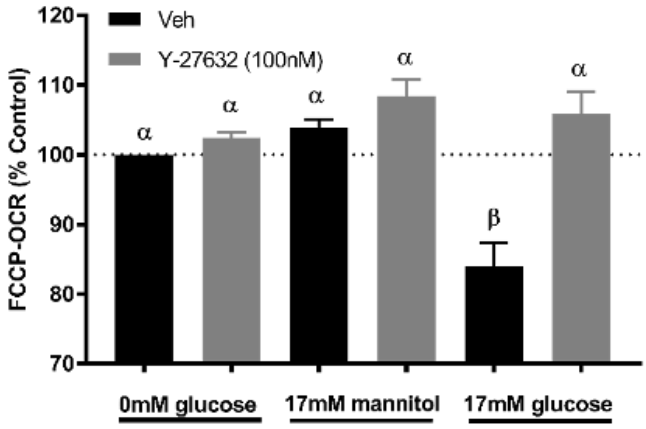
G


FCCP
Veh
CCG-1432
OCR (pMoles/min)
500
400
300
200
0mM glucose
17mM mannitol
17mM glucose

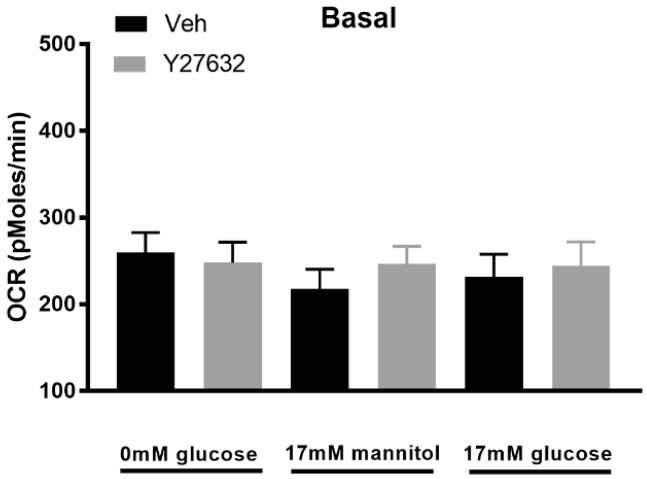
H


Veh
Y-27632 (100nM)
Basal-OCR (% Control)
120
110
100
90
80
70
α
0mM glucose
17mM mannitol
17mM glucose

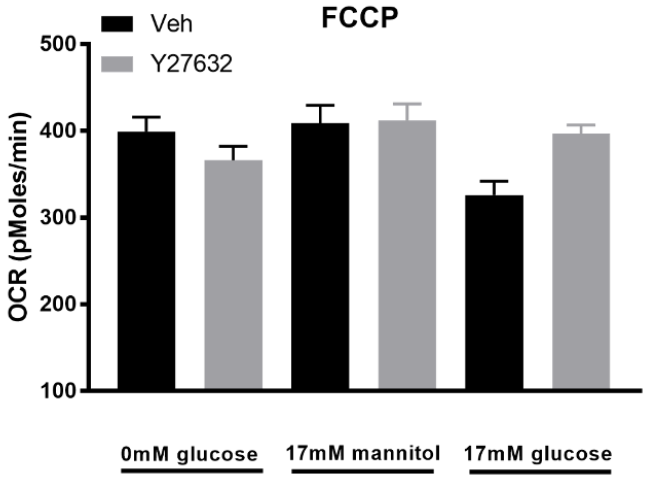
I


Veh
Y-27632 (100nM)
FCCP-OCR (% Control)
120
110
100
90
80
70
α
β
0mM glucose
17mM mannitol
17mM glucose

J


Basal
Veh
Y27632
OCR (pMoles/min)
500
400
300
200
100
0mM glucose
17mM mannitol
17mM glucose

K


FCCP
Veh
Y27632
OCR (pMoles/min)
500
400
300
200
100
0mM glucose
17mM mannitol
17mM glucose

L

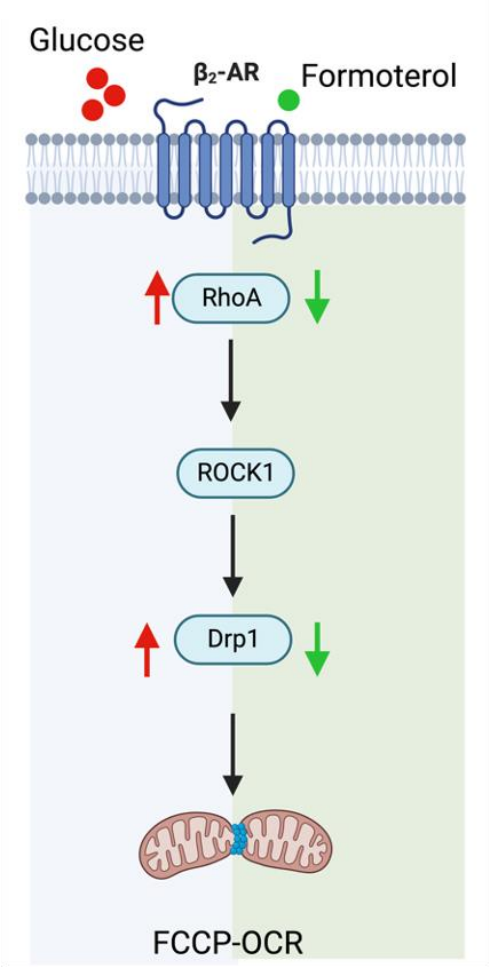
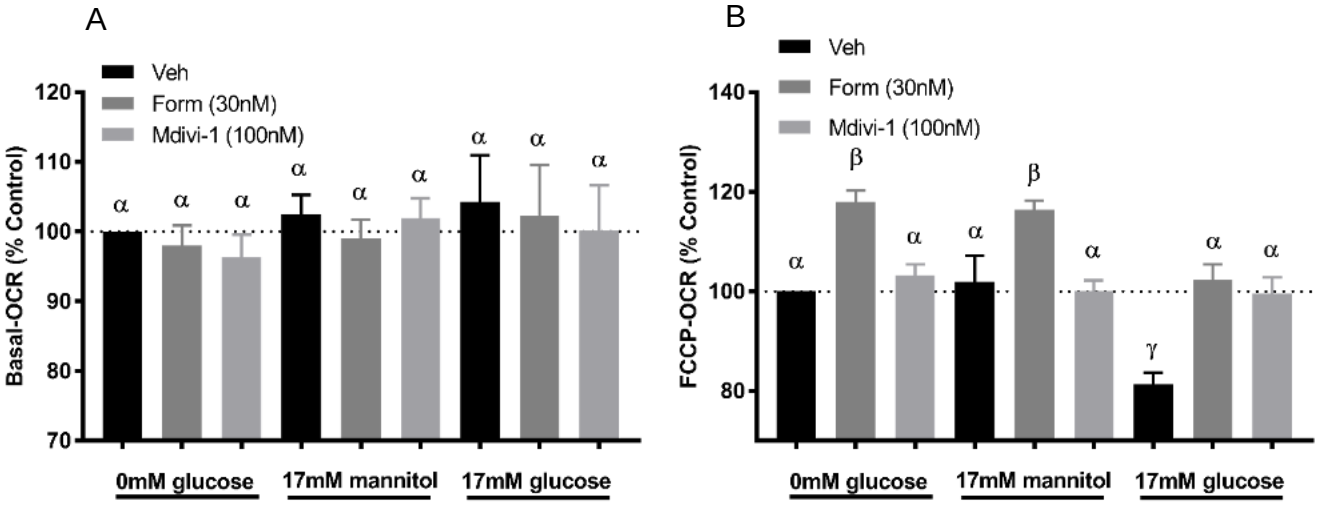


Figure 3.2. Formoterol, CCG-1423 and Y-27632 restore RhoA and Drp1 activity and mitochondrial function in RPTC. CCG-1423 (300nM) and Y-27632 (100nM) are RhoA and ROCK1 inhibitors, respectively. RPTC were grown in 0mM glucose, 17mM mannitol or 17mM glucose for 96 hr. At 72 hr, cells were treated with either vehicle (0.1% DMSO), formoterol, CCG-1423 or Y-27632 for 24 hr. Cells were harvested and proteins were subjected to immunoblot analysis of (A) GTP-bound RhoA and total RhoA and (B) and (C) GTP-bound Drp1 and total Drp1. Seahorse XF96 Analyzer was used to measure oxygen consumption under the same conditions and co-treated with either CCG-1423 (D) basal-OCR measurements and (E) FCCP-OCR, or Y-27632 (F) basal-OCR and (G)

FCCP-OCR. Data represented as mean±SEM, n=6. Statistical significance was determined by two-way ANOVA and Tukey-Kramer post-hoc test. Different Greek letters signify statistical differences.


A
Veh
Form (30nM)
Mdivi-1 (100nM)
Basal-OCR (% Control)
120
110
100
90
80
70
α
0mM glucose
17mM mannitol
17mM glucose
B
Veh
Form (30nM)
Mdivi-1 (100nM)
FCCP-OCR (% Control)
140
120
100
80
α
β
γ
0mM glucose
17mM mannitol
17mM glucose

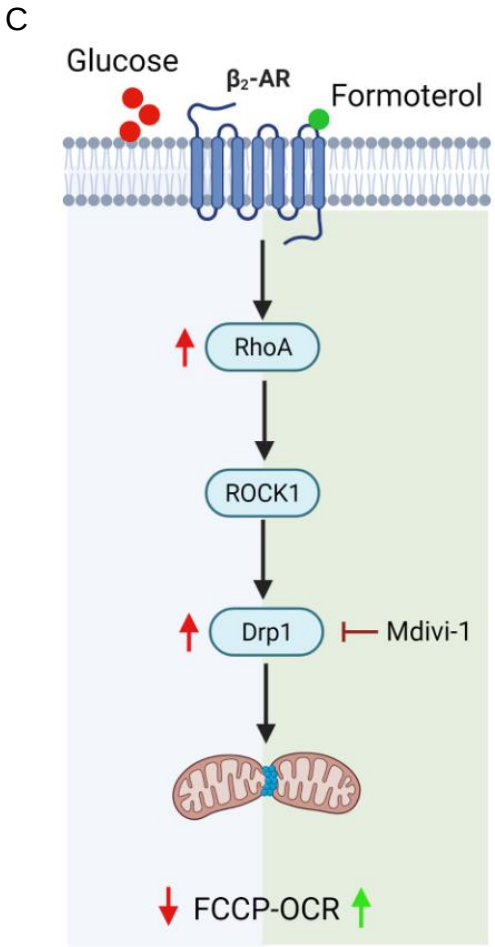
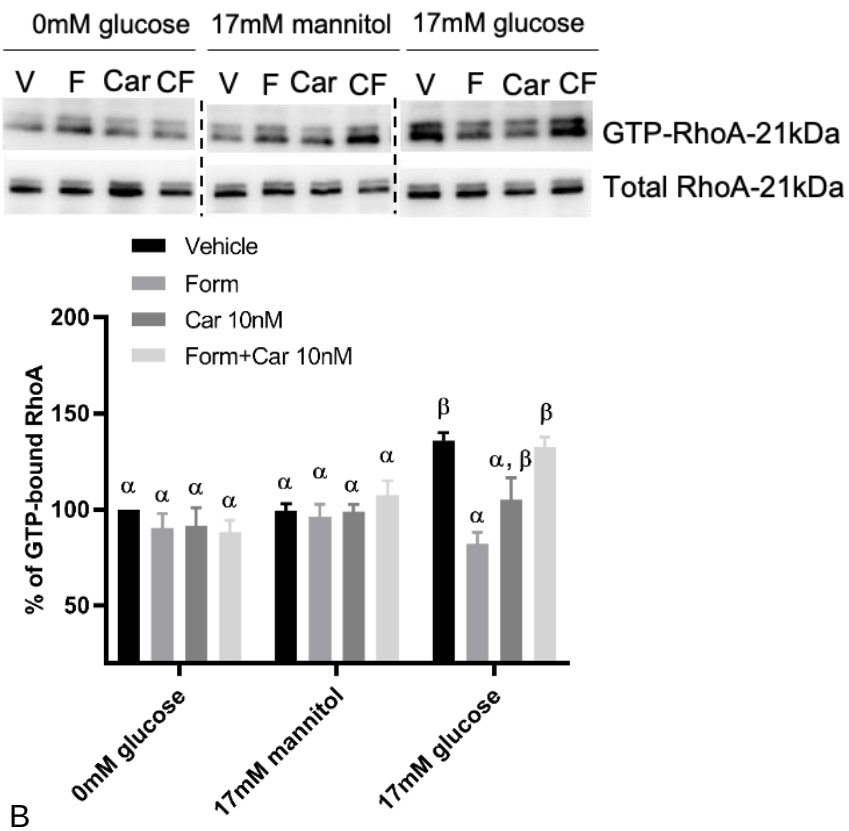

C
Glucose
β2-AR
Formoterol
RhoA
ROCK1
Drp1
Mdivi-1
FCCP-OCR

Figure 3.3. **Formoterol and Mdivi-1 restore mitochondrial function in glucose treated RPTC.** RPTC were grown in 0mM glucose, 17mM mannitol or 17mM glucose for 96 hr. At 72 hr, cells were co-treated with either vehicle (0.1% DMSO), formoterol (30nM) or Mdivi-1 (100nM) for 24 hr. Seahorse XF96 Analyzer was used to measure (A) basal-OCR or (B) FCCP-OCR. Data represented as mean±SEM, n=6. Statistical significance was determined by two-way ANOVA and Tukey-Kramer post-hoc test. Different Greek letters signify statistical differences.

A


0mM glucose
17mM mannitol
17mM glucose
V F Car CF V F Car CF V F Car CF
GTP-RhoA-21kDa
Total RhoA-21kDa
Vehicle
Form
Car 10nM
Form+Car 10nM
% of GTP-bound RhoA
200
150
100
50
0mM glucose
17mM mannitol
17mM glucose

B

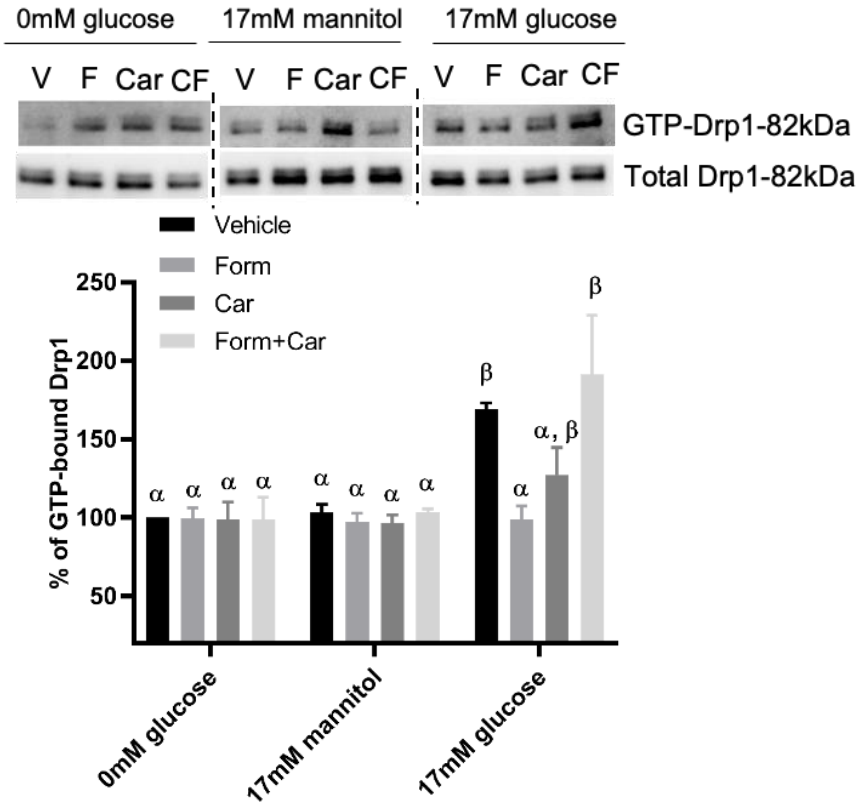
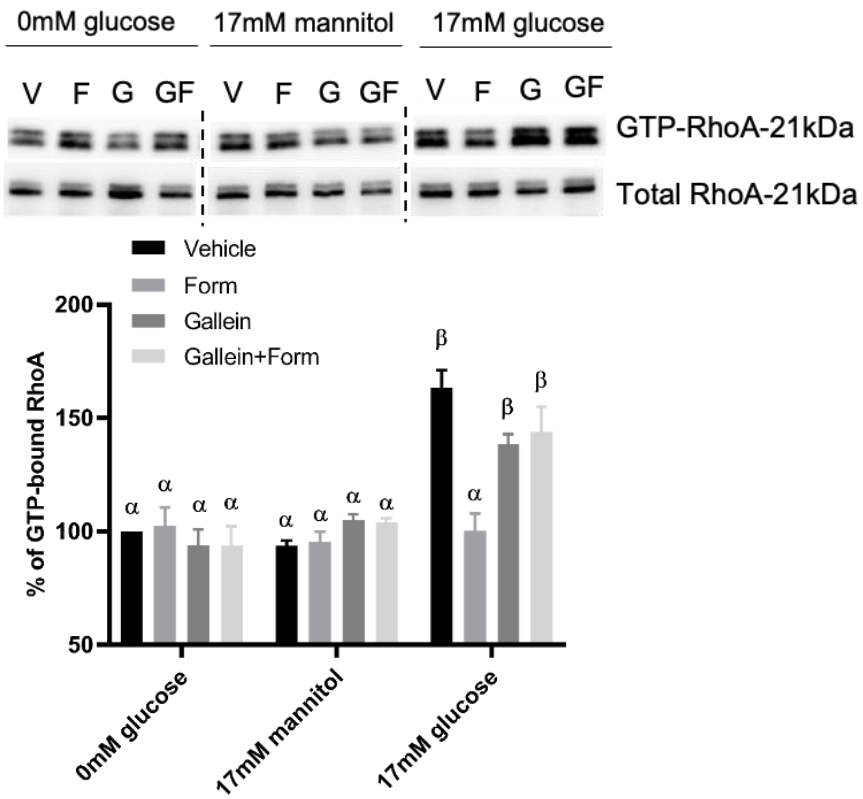
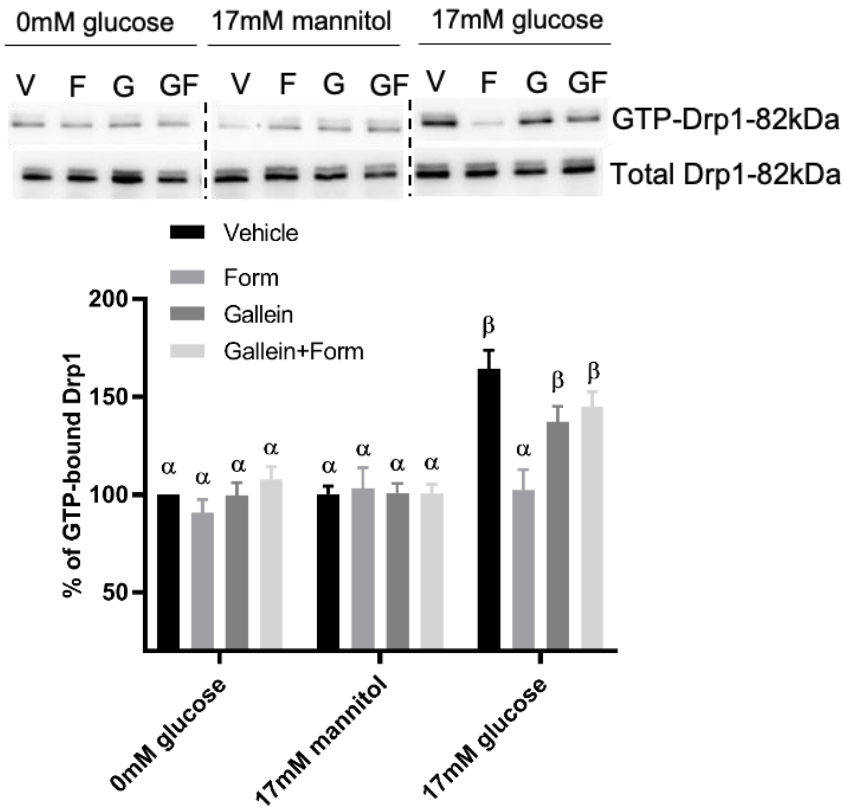

0mM glucose
17mM mannitol
17mM glucose
V F Car CF V F Car CF V F Car CF
GTP-Drp1-82kDa
Total Drp1-82kDa
Vehicle
Form
Car
Form+Car
% of GTP-bound Drp1
250
200
150
100
50
0mM glucose
17mM mannitol
17mM glucose

Figure 3.4. **Carvedilol blocks the effect of formoterol on RhoA and Drp1 activity.** RPTC were grown in 0mM glucose, 17mM mannitol or 17mM glucose for 96 hr. At 72 hr, cells were co-treated with either vehicle (0.1% DMSO), formoterol (30nM), carvedilol (10nM) or carvedilol+formoterol for 24 hr. Cells were harvested and proteins were subjected to immunoblot analysis of (A) GTP-bound RhoA and total RhoA and (B) GTP-bound Drp1 and total Drp1. Data represented as mean±SEM, n=6. Statistical significance was determined by two-way ANOVA and Tukey-Kramer post-hoc test. Different Greek letters signify statistical differences.

A


0mM glucose
17mM mannitol
17mM glucose
V F G GF V F G GF V F G GF
GTP-RhoA-21kDa
Total RhoA-21kDa
Vehicle
Form
Gallein
Gallein+Form
% of GTP-bound RhoA
200
150
100
50
α
β
0mM glucose
17mM mannitol
17mM glucose

B


0mM glucose
17mM mannitol
17mM glucose
V F G GF V F G GF V F G GF
GTP-Drp1-82kDa
Total Drp1-82kDa
Vehicle
Form
Gallein
Gallein+Form
% of GTP-bound Drp1
200
150
100
50
α
β
0mM glucose
17mM mannitol
17mM glucose

C

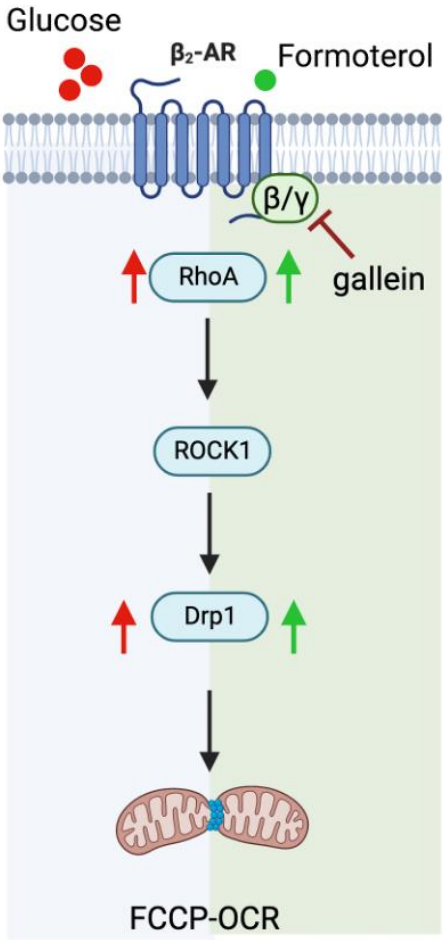
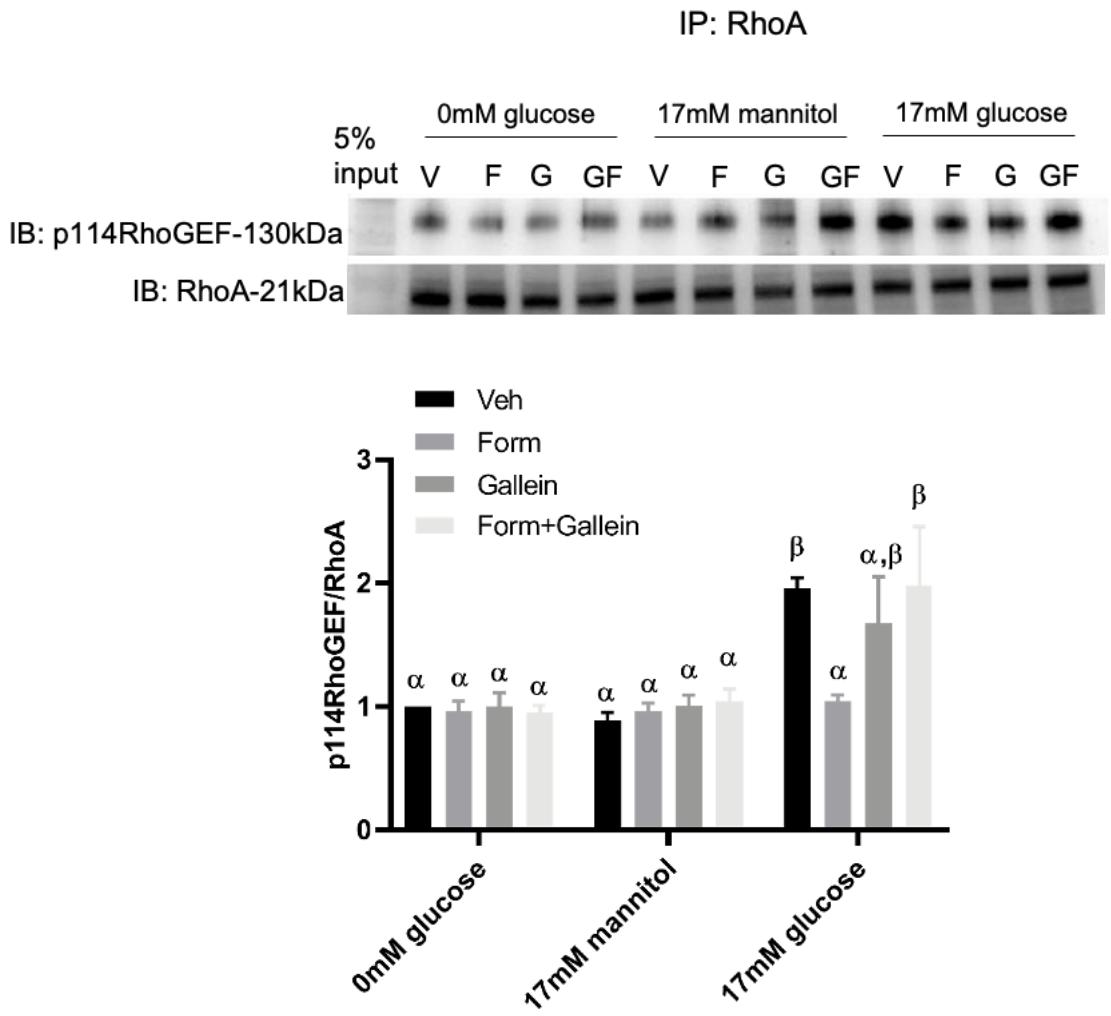


Figure 3.5. **Gallein blocks the effect of formoterol on RhoA and Drp1 activity**. RPTC were grown in 0mM glucose, 17mM mannitol or 17mM glucose for 96 hr. At 72 hr, cells were co-treated with either vehicle (0.1% DMSO), formoterol (30nM), gallein (100nM) or gallein+formoterol. Cells were harvested and proteins were subjected to immunoblot analysis of (A) GTP-bound RhoA and total RhoA and (B) GTP-bound Drp1 and total Drp1. Data represented as mean±SEM, n=6. Statistical significance was determined by two-

way ANOVA and Tukey-Kramer post-hoc test. Different Greek letters signify statistical differences.


IP: RhoA
0mM glucose
17mM mannitol
17mM glucose
5%
input
V F G GF V F G GF V F G GF
IB: p114RhoGEF-130kDa
IB: RhoA-21kDa
Veh
Form
Gallein
Form+Gallein
p114RhoGEF/RhoA
0
1
2
3
α
β
α,β
0mM glucose
17mM mannitol
17mM glucose

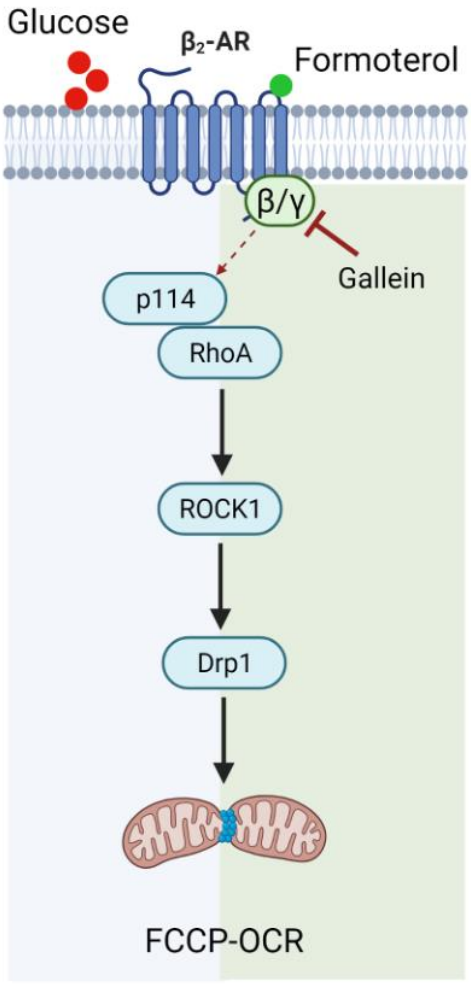
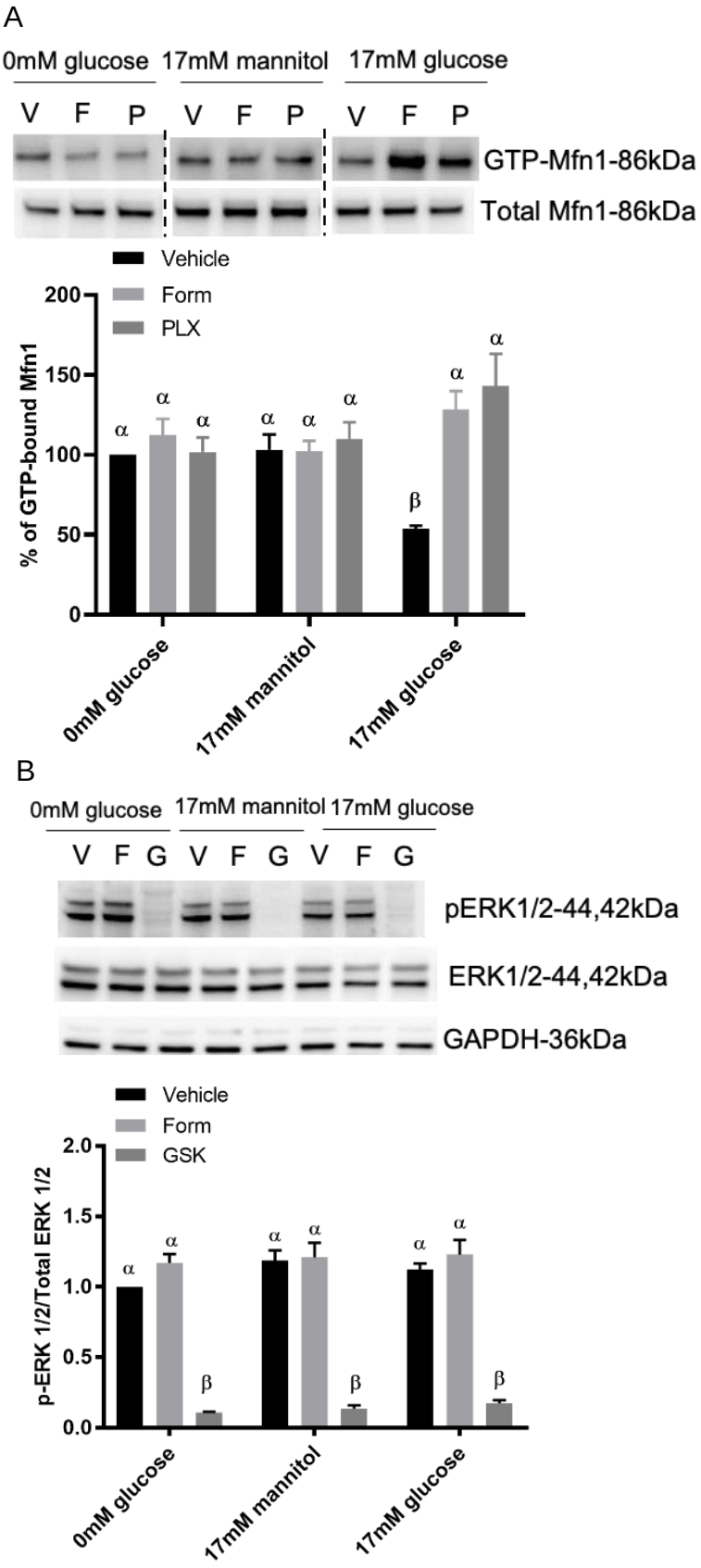
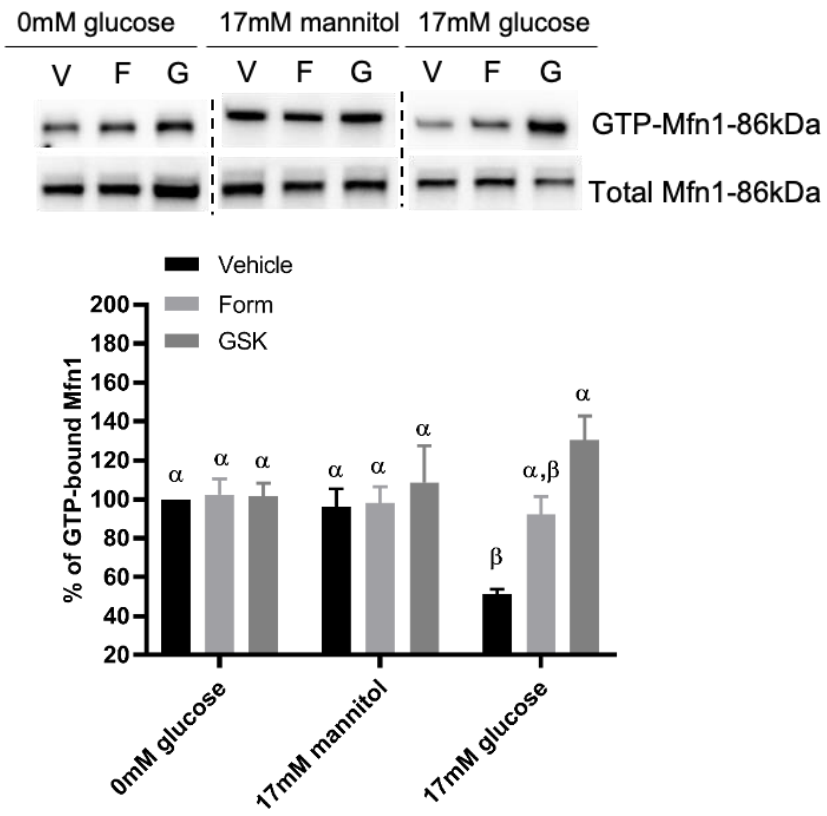


Figure 3.6. **Formoterol blocks the interaction between p114RhoGEF and RhoA.** RPTC were grown in 0mM glucose, 17mM mannitol or 17mM glucose for 96 hr. At 72 hr, cells were co-treated with either vehicle (0.1% DMSO), formoterol (30nM), gallein (100nM) or gallein+formoterol. p114RhoGEF and RhoA were measured by immunoblot after immunoprecipitation of RhoA. Data represented as mean±SEM, n=6. Statistical significance was determined by two-way ANOVA and Tukey-Kramer post-hoc test. Different Greek letters signify statistical differences.


A
0mM glucose
17mM mannitol
17mM glucose
V F P V F P V F P
GTP-Mfn1-86kDa
Total Mfn1-86kDa
Vehicle
Form
PLX
% of GTP-bound Mfn1
200
150
100
50
0
α
β
0mM glucose
17mM mannitol
17mM glucose
B
0mM glucose
17mM mannitol
17mM glucose
V F G V F G V F G
pERK1/2-44,42kDa
ERK1/2-44,42kDa
GAPDH-36kDa
Vehicle
Form
GSK
p-ERK 1/2/Total ERK 1/2
2.0
1.5
1.0
0.5
0.0
α
β
0mM glucose
17mM mannitol
17mM glucose

C


0mM glucose
17mM mannitol
17mM glucose
V F G V F G V F G
GTP-Mfn1-86kDa
Total Mfn1-86kDa
Vehicle
Form
GSK
% of GTP-bound Mfn1
200
180
160
140
120
100
80
60
40
20
α α α α α α α α,β α
β
0mM glucose
17mM mannitol
17mM glucose

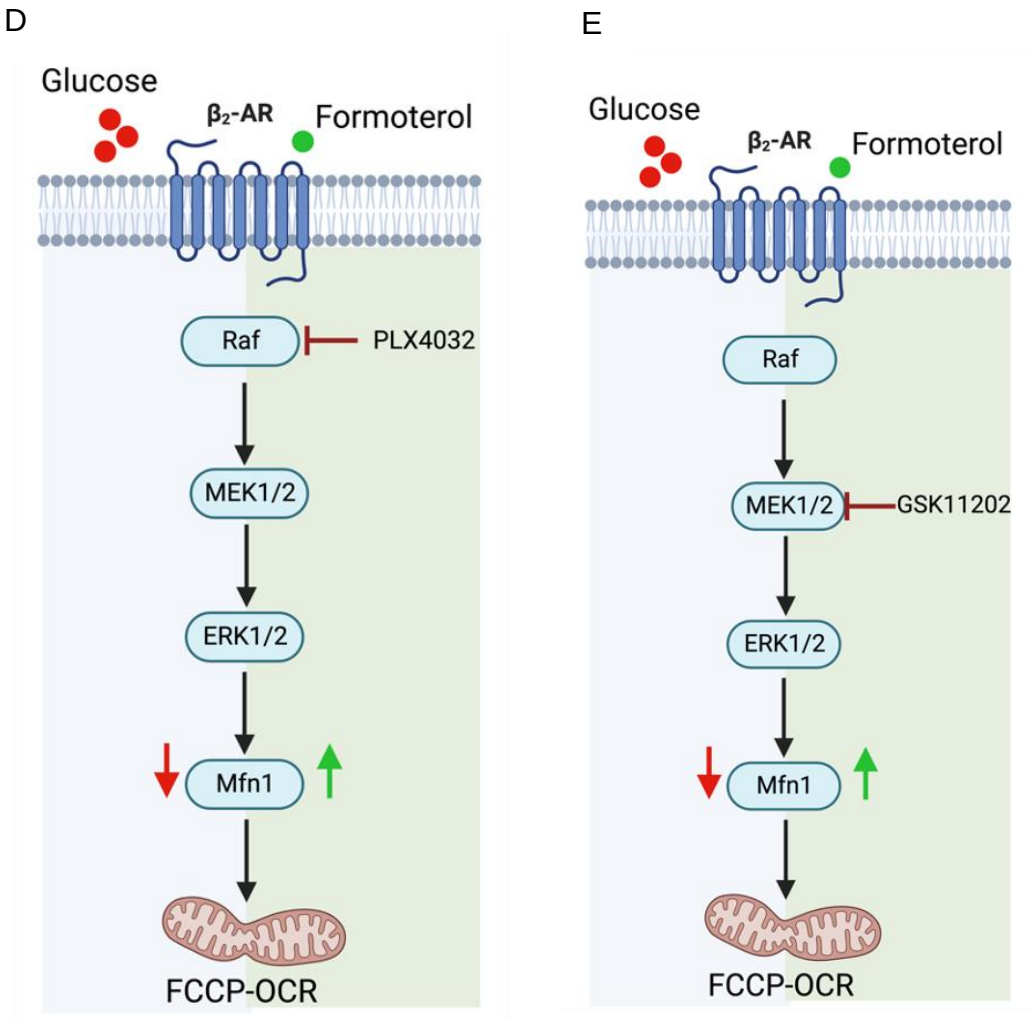
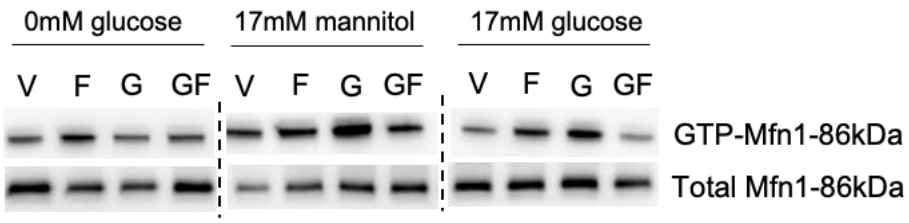
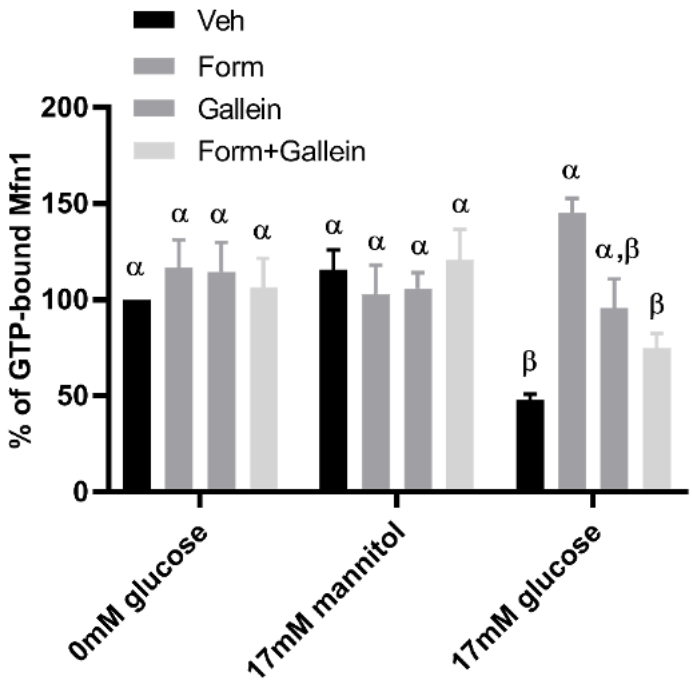


Figure 3.7. **Formoterol, PLX4032 and GSK11202 restore Mfn1 activity in glucose treated RPTC**. RPTC were grown in 0mM glucose, 17mM mannitol or 17mM glucose for 96 hr. At 72 hr, cells were co-treated with either vehicle (0.1% DMSO), formoterol (30nM), PLX4032 (30nM) or GSK11202 (10nM). Harvested proteins were subjected to immunoblot analysis of (A) and (C) GTP-bound and total Mfn1 and (B) pERK1/2 or total ERK1/2. Data represented as mean±SEM, n=6. Statistical significance was determined by two-way ANOVA and Tukey-Kramer post-hoc test. Different Greek letters signify statistical differences.


0mM glucose
17mM mannitol
17mM glucose
V F G GF V F G GF V F G GF
GTP-Mfn1-86kDa
Total Mfn1-86kDa


Veh
Form
Gallein
Form+Gallein
% of GTP-bound Mfn1
200
150
100
50
0
α
α
α
α
α
α
α
α
β
α
α,β
β
0mM glucose
17mM mannitol
17mM glucose

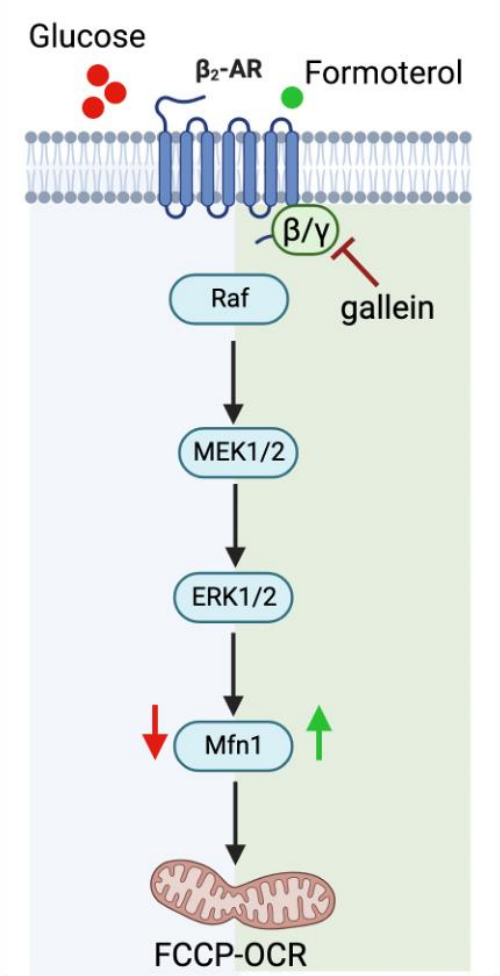


Figure 3.8. **Gallein blocks the effect of formoterol on Mfn1 activity in glucose treated RPTC**. RPTC were grown in 0mM glucose, 17mM mannitol or 17mM glucose for 96 hr. At 72 hr, cells were co-treated with either vehicle (0.1% DMSO), formoterol (30nM), gallein (100nM) or gallein+formoterol. Cells were harvested and proteins were subjected to immunoblot analysis of GTP-bound and total Mfn1 after treatment with gallein. Data represented as mean±SEM, n=6. Statistical significance was determined by two-way ANOVA and Tukey-Kramer post-hoc test. Different Greek letters signify statistical differences.

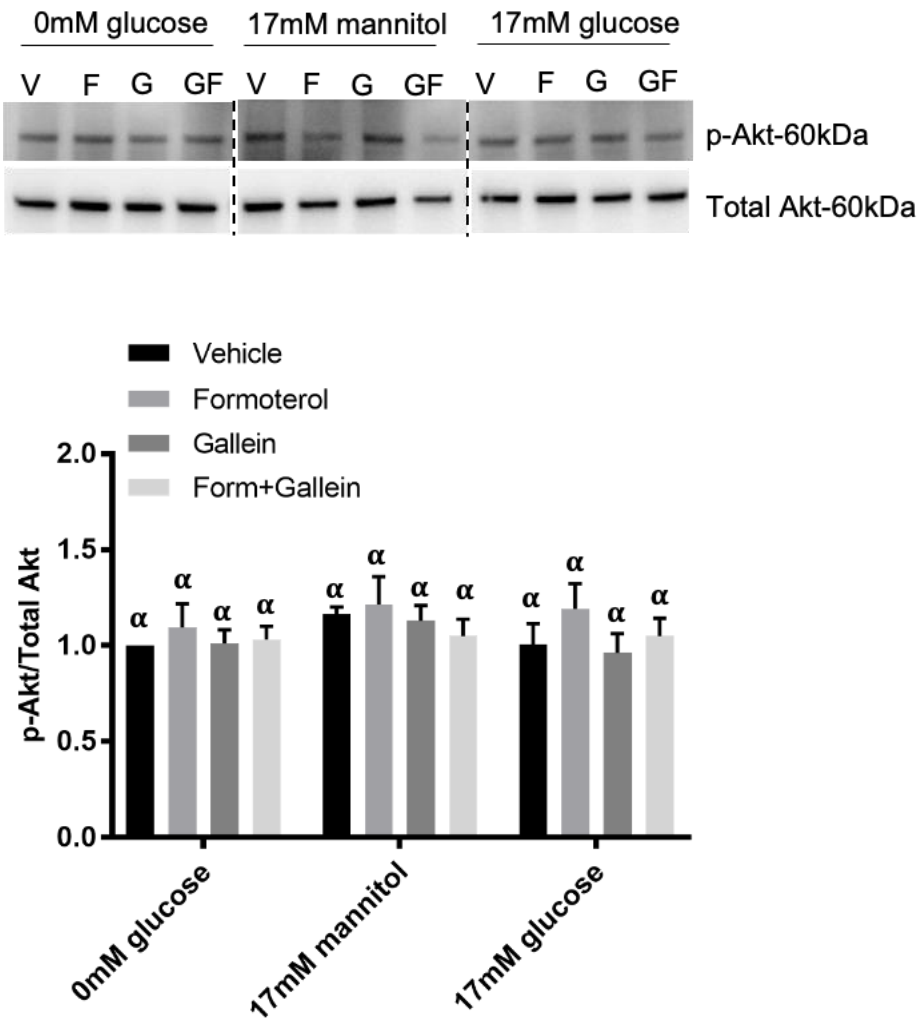


Figure 3.9. **Formoterol and gallein have no effect on Akt phosphorylation in glucose treated RPTC.** RPTC were grown in 0mM glucose, 17mM mannitol or 17mM glucose for 96 hr. At 72 hr, cells were co-treated with either vehicle (0.1% DMSO), formoterol (30nM), gallein (100nM) or gallein+formoterol. Cells were harvested and proteins were subjected to immunoblot analysis of GTP-bound and total Mfn1 after treatment with gallein. Data represented as mean±SEM, n=6. Statistical significance

was determined by two-way ANOVA and Tukey-Kramer post-hoc test. Different Greek letters signify statistical differences.

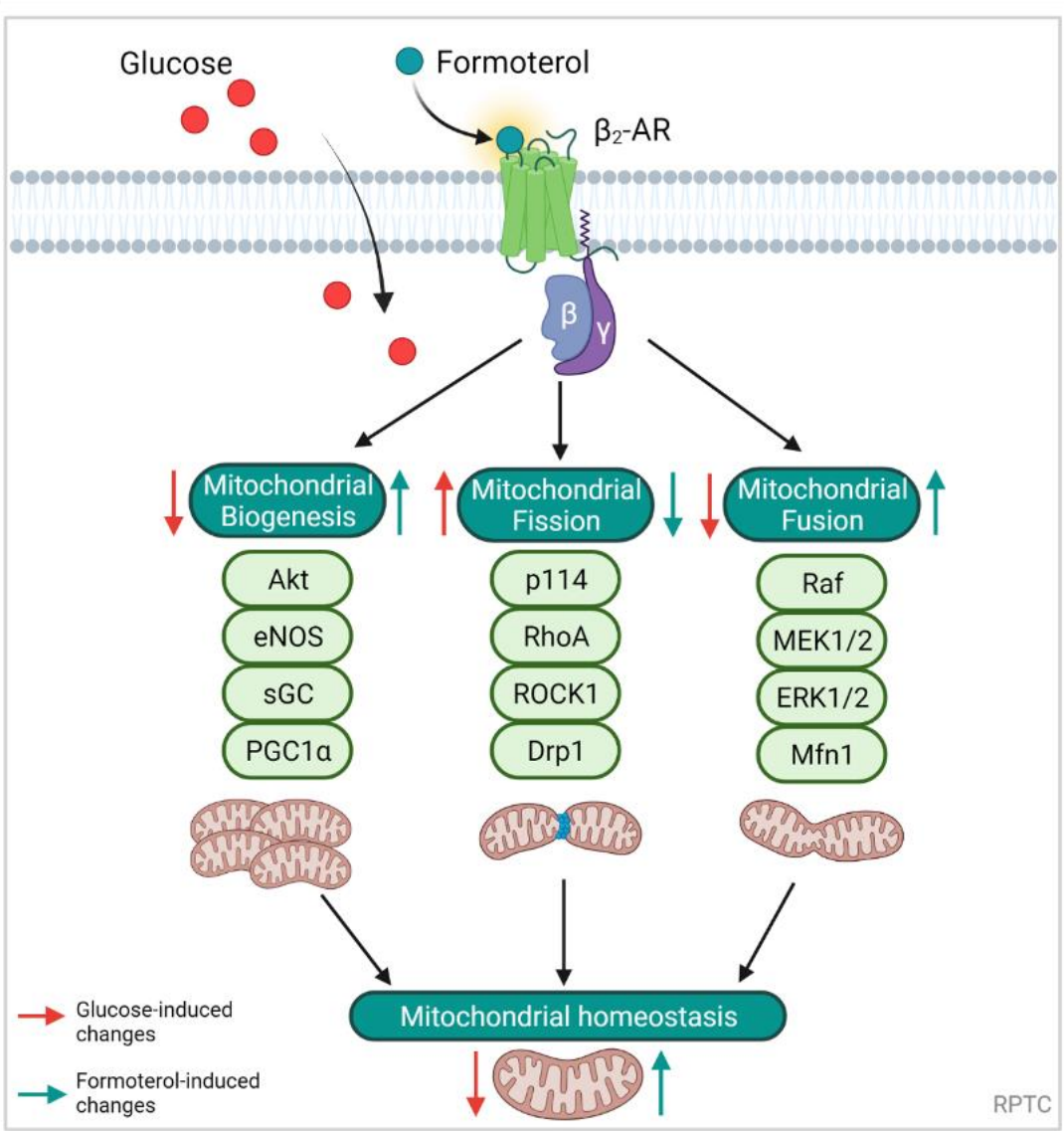


Figure 3.10. Formoterol restores mitochondrial homeostasis through activation of three separate and integrated mechanisms. Glucose increases RhoA/ROCK1-

mediated activation of Drp1 leading to increased fission, increases Raf/MEK1/2/ERK1/2 signaling leading to decreased Mfn1 activation and in turn decreased mitochondrial fusion. Glucose also decreases mitochondrial biogenesis. Formoterol restores these pathways through activation of the G$\beta\gamma$ subunit of the β2-AR. These pathways work simultaneously to regulate mitochondrial homeostasis in RPTC.

Chapter 4:

Conclusions and Future Research

4.1 Summary of Relevant Findings

Diabetes is a prevalent metabolic disease that contributes to ~50% of all ESRD and has limited treatment options. Hypertension and hyperglycemia are major drivers of DKD, and mounting evidence shows that hyperglycemia is a contributing factor in renal mitochondrial dysfunction. Mitochondrial dysfunction as a result of hyperglycemia is characterized by decreased mitochondrial biogenesis, fusion, increased fission and altered ETC activity. These dysfunctions ultimately lead to an imbalance in mitochondrial homeostasis and negatively impact normal physiological functions. In the case of DKD, recent studies have pinpointed mitochondrial dysfunction as a driving factor in the development and progression of the disease and have demonstrated that restoring aspects of mitochondrial function, such as mitochondrial dynamics, can lead to improved disease progression and intriguingly, reverse several hallmark features of DKD. Pharmacological agonists such as formoterol have been demonstrated to exert beneficial mitochondrial effects, leading to improved renal function in both acute and chronic diseases. Finally, we suggest that repurposing FDA-approved therapeutics, such as formoterol, may be beneficial for the treatment of DKD.

Our initial studies in RPTC demonstrated that chronic exposure to high glucose induced mitochondrial dysfunction. Glucose-induced injury led to increased phosphorylation of the mitochondrial fission protein Drp1 and expression of ETC complex subunits, decreased expression of the mitochondrial fusion protein Mfn1, further leading

to decreased ETC complex III activity as well as maximal mitochondrial respiration. When RPTC were co-treated with formoterol, these changes were restored. The altered expression of mitochondrial proteins observed in RPTC in response to high glucose were almost exactly mimicked in renal cortical tissue of diabetic db/db mice. Similarly to what was observed *in vitro*, formoterol restored these changes.

It is known that Drp1 mitochondrial translocation and activity is dependent on context and stimulus dependent phosphorylation. There have been quite a few conflicting studies which report that phosphorylation at Ser637 can lead to both activation and de-activation. However, we establish here that glucose-induced phosphorylation of Ser637 leads to increased Drp1-GTP activity. Upon further investigation of the mechanisms involved in glucose-induced changes in Drp1 activity, we show that RhoA/ROCK1 is responsible for this increase in activity. Importantly, it was shown that this pathway is activated as a result of increased interaction between RhoA and p114RhoGEF. Formoterol prevented this interaction by activating Gβγ and leading to decreased RhoA and Drp1 activity in RPTC.

From our initial studies, we determined that Mfn1 expression was decreased in high glucose. To verify that altered Mfn1 expressed also led to functional changes, it was demonstrated that GTP-bound Mfn1 was also decreased in high glucose. Furthermore, we identified Raf/MEK1/2/ERK1/2 as upstream regulators of the mitochondrial fusion protein Mfn1 in RPTC exposed to high glucose. When pharmacological inhibitors were used to block this pathway, Mfn1 activity was restored. Importantly, formoterol restored Mfn1 activity via activation of the Gβγ subunit of the β2-AR. From these studies, we

determined that formoterol activated three distinct, separate yet integrated signaling pathways in RPTC to regulate mitochondrial homeostasis.

4.2 Future Research

The work in this dissertation identified alterations of the proteins and signaling pathways involved in mitochondrial dynamics. One of the limitations of these studies is that real-time imaging of mitochondrial dynamics was not assessed. In order to determine that the observed changes also correlate with changes in mitochondrial morphology, future studies should utilize confocal microscopy to observe live cell changes in mitochondrial dynamics.

Metabolomic changes are an important component in the development and progression of DKD and are often present with mitochondrial dysfunction (42, 166). This evidence suggests there is a link between mitochondrial dysfunction, altered metabolism and renal dysfunction in DKD. To date, research shows that altered metabolism leads to mitochondrial dysfunction through a number of ways. Altered metabolism and increased metabolic flux can lead to activation of RAS, generating mitochondrial oxidative damage (158-161). In addition, these changes in metabolism can increase the number of post-translational modifications, subsequently altering enzymatic activity of proteins involved in the TCA cycle and ATP production (162). Interestingly, metabolic flux in diabetic mice is associated with defective mitophagy and bioenergetics (163-165).

Given the evidence providing a link between mitochondrial dysfunction and metabolism, it would be worthwhile to study the effects of formoterol on metabolic

enzymes in early hyperglycemia. Prior studies have identified that in early DKD, metabolic flux is increased (42). Since it has also been established that mitochondrial dysfunction is also present in the early stages in hyperglycemia in our studies and in others, it is clear that both dysfunctions take place simultaneously. In our studies, it has been demonstrated that formoterol restores mitochondrial function by inducing MB, restoring the balance in mitochondrial dynamics and homeostasis, and additionally has effects on mitochondrial bioenergetics. Therefore, it is possible that formoterol also has effects on metabolism and potentially metabolic flux. Based on these results, we performed a study using diabetic db/db mice (13 weeks) and treated with formoterol to evaluate the effects of formoterol on early metabolic changes in DKD. This study has not yet been completed, however the data will yield further insight into the mechanisms through which formoterol works to restore mitochondrial function in DKD.

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