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The β2-adrenoceptor agonist formoterol restores mitochondrial homeostasis in glucose-induced renal proximal tubule injury through separate integrated pathways

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Abstract

Background and Purpose: Mitochondrial dysfunction is a driving factor in the development and progression of diabetic kidney disease (DKD). Our laboratory discovered that the ?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. Experimental Approach: Using primary cultures of renal proximal tubule cells (RPTC) exposed to high glucose, we investigated the role of glucose on RhoA/ROCK1/Drp1 and Raf/MEK1/2/ERK1/2/Mfn1 signaling pathways using pharmacological inhibitors, and the effect of formoterol on these pathways. Key Results: In high glucose, RhoA became hyperactive, leading to ROCK1-induced activation of Drp1. Using pharmacological inhibitors, formoterol signals through G?? subunits of the ?2-adrenoceptor to decrease RhoA/ROCK1-mediated activation of Drp1. Formoterol restored this pathway by preventing the interaction of RhoA with the guanine nucleotide exchange factor p114RhoGEF. Inhibition of RhoA/ROCK1/Drp1 restored maximal mitochondrial respiration. Formoterol also restored Mfn1 through a separate G??-dependent mechanism composed of Raf/MEK1/2/ERK1/2/Mfn1. RPTC exposed to high glucose exhibited decreased Mfn1 activation, which was restored with formoterol. Pharmacological inhibition of G??, Raf and MEK1/2 also restored Mfn1 activity. Conclusion and Implications: 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 integrative pathways that promote mitochondrial biogenesis, decreased fission and increased fusion in RPTC, supporting its potential as a therapeutic for DKD.

Title

The β_2-adrenoceptor agonist formoterol restores mitochondrial homeostasis in glucose-induced renal proximal tubule injury through separate integrated pathways

Short running title

Formoterol restores mitochondrial homeostasis

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Experimental Approach: Using primary cultures of renal proximal tubule cells (RPTC) exposed to high glucose, we investigated the role of glucose on RhoA/ROCK1/Drp1 and Raf/MEK1/2/ERK1/2/Mfn1 signaling pathways using pharmacological inhibitors, and the effect of formoterol on these pathways.

Key Results: In high glucose, RhoA became hyperactive, leading to ROCK1-induced activation of Drp1. Using pharmacological inhibitors, formoterol signals through G$\beta\gamma$ subunits of the β_2-adrenoceptor to decrease RhoA/ROCK1-mediated activation of Drp1. Formoterol restored this pathway by preventing the interaction of RhoA with the guanine nucleotide exchange factor p114RhoGEF. Inhibition of RhoA/ROCK1/Drp1 restored maximal mitochondrial respiration. Formoterol also restored Mfn1 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. Pharmacological inhibition of G$\beta\gamma$, Raf and MEK1/2 also restored Mfn1 activity.

Conclusion and Implications: 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 integrative pathways that promote mitochondrial biogenesis, decreased fission and increased fusion in RPTC, supporting its potential as a therapeutic for DKD.

Abbreviations

Diabetic kidney disease (DKD)

End-stage renal disease (ESRD)

Renal proximal tubule cells (RPTC)

Mitochondrial biogenesis (MB)

Key words

Diabetic kidney disease (DKD), mitochondria, formoterol, β_2-adrenoceptor, renal proximal tubule

Introduction

Diabetic kidney disease (DKD) is a prevalent metabolic disease and is the most common cause of end stage renal disease (ESRD) (Fu et al., 2019). 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.

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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 (Vallon, 2011). 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 (Abbate & Remuzzi, 1999; Bohle et al., 1991; Cleveland et al., 2020; Forbes & Thorburn, 2018; Huang & Preisig, 2000; Vallon & Verma, 2021). 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 (Bhargava & Schnellmann, 2017; Lynch et al., 2018). 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 (Lee et al., 2017).

In addition, studies report that mitochondrial dynamics is altered in diabetic db/db mice and in glomerular podocytes exposed to high glucose (Ayanga et al., 2016; Galvan et al., 2019). 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 (Audzeyenka et al., 2021). 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 (Cleveland et al., 2020). 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) (W. Wang et al., 2012). Furthermore, separate studies show that Ras homolog family member A (RhoA) is responsible for ROCK1 activation and subsequent Drp1 phosphorylation (Brand et al., 2018). 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 (Pyakurel et al., 2015). In addition, it has been demonstrated that Raf, the upstream activator of MEK1/2 is also upregulated in the presence of glucose (Trumper et al., 2005).

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 (Jesinkey et al., 2014; Wills et al., 2012). 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 (Cameron et al., 2017). Based on these findings, our hypothesis was formoterol signals through the G$\beta\gamma$ 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.

Methods

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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 & Schnellmann, 1995, 1996). 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.

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°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°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.

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°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°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.

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, pAkt (#9271), Akt (#9272) 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.

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 1.6×10^4 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

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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).

Data and 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). The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology. Technical replicates were used to ensure the reliability of single values.

Materials

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.

Results

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 was used to determine Drp1 and RhoA activity. RPTC exposed to high glucose had increased GTP-bound RhoA (Fig 1A) and Drp1 (Fig 1B) 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 2A). In addition, treatment with CCG-1423 prevented glucose-induced activity of Drp1 (Fig 2B). Seahorse XF96 analysis was used to determine the effect of RhoA inhibition on FCCP-OCR, a marker of MB and maximal electron transport chain activity. Glucose exposure did not change basal respiration in any treatment group (Fig 2D). 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 2E).

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 2C). Similarly to what was observed after RhoA inhibition, inhibition of ROCK1 had no effect on basal-OCR under any conditions (Fig 2F) and restored maximal mitochondrial respiration to the same level as controls (Fig 2G). 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 3A). Formoterol increased FCCP-OCR in 0mM glucose and mannitol controls compared to vehicle, whereas Mdivi-1 did not (Fig 3B). However, RPTC grown in high glucose, both formoterol and Mdivi-1 treatment restored FCCP-OCR.

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) was used 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 4A and B). We previously discovered that formoterol activates the G$\beta\gamma$ subunit of the β_2-adrenoceptor to

induce MB (Cameron et al., 2017). Therefore, to determine whether formoterol works through Gβγ signaling to regulate Drp1. RPTC were grown in the presence or absence of glucose with the Gβγ inhibitor gallein. Gallein blocked the ability of formoterol to restore both RhoA and Drp1 activity (Fig 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 may regulate RhoA activity. Among these RhoGEFs, we found that the interaction between RhoA and p114RhoGEF was increased in the presence of glucose (Fig 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.

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, the same GTP pulldown assay to measure Mfn1 activity was used. In RPTC treated with vehicle in the presence of high glucose, Mfn1 activity was decreased compared to 0mM glucose and mannitol controls (Fig 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 7B). In addition, both GSK11202 and formoterol treatment prevented the decrease in Mfn1 activity compared to 0mM glucose and 17mM mannitol controls (Fig 7C).

To determine whether the Raf/MEK1/2/ERK1/2/Mfn1 pathway is also regulated by formoterol activation of Gβγ, gallein was used 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 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 RPTC in all groups (Fig 9).

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 (Ayanga et al., 2016; Cleveland et al., 2020; Galvan et al., 2019; S. Wang et al., 2012). Prior studies have identified that ROCK1 is an activator of Drp1 (W. Wang et al., 2012). Separate studies have identified that RhoA also leads to increased Drp1 phosphorylation and translocation to the mitochondria in a ROCK1-dependent manner (Brand et al., 2018). Aside from RhoA/ROCK1, Drp1 is also phosphorylated by protein kinase D (PKD) at Ser637 to initiate mitochondrial fission (Jhun et al., 2018). 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 (Chang & Blackstone, 2007).

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 Gβγ 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. Ultimately, formoterol blocks glucose-induced DRP 1 activity Despite this finding, it is still unclear exactly how Gβγ blocks the interaction between p114RhoGEF and RhoA.

As previously reported, high glucose decreases maximal mitochondrial respiration (Cleveland et al., 2020). Using pharmacological inhibitors targeting RhoA, ROCK1 and Drp1, maximal mitochondrial respiration was

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restored, indicating that in addition to its role in mitochondrial fission, Drp1 may also play a role in regulating mitochondrial respiration. A study evaluating the effects of Drp1 inhibition on mitochondrial function found that inhibition of endogenous Drp1 suppressed maximal respiration. Furthermore, the authors showed that Drp1 is responsible for maintaining mitochondrial respiration and bioenergetics independently of its role in mitochondrial fission (Zhang et al., 2017), indicating that Drp1 regulates mitochondrial function in addition to its role in maintaining mitochondrial morphology.

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 (Duan & Cobb, 2010; Khoo & Cobb, 1997; Trumper et al., 2005). Pyakurel et al. demonstrated that MEK1/2/ERK1/2 phosphorylates Mfn1 to modulate its activity as a mechanism of apoptosis regulation (Pyakurel et al., 2015). Based on these studies, there is evidence linking MEK1/2/ERK1/2 to Mfn1 activation. Despite this knowledge, the signaling pathway has not yet been elucidated in renal cell types exposed to hyperglycemic conditions. High glucose decreased Mfn1 and pharmacological inhibitors of Raf and MEK1/2 restored Mfn1 activity. 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 (Goldsmith & Dhanasekaran, 2007; Ito et al., 1995), 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 (Whitaker et al., 2016). It has previously been determined that formoterol activation of Gβγ leads to PI3K-dependent activation of Akt/eNOS/sGC in RPTC (Cameron et al., 2017), and activation of this pathway leads to increased PGC1α and 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 MB and mitochondrial homeostasis in RPTC in response to high glucose injury (graphical abstract). 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 (O'Hayre et al., 2017). 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 are limited and indirect drugs that decrease 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 (Vazquez-Medina et al., 2013). Another antagonist, losartan, provided renal mitochondrial protection from oxidative injury (Katyare & Satav, 2005). 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 (He et al., 2022). 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

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to its beneficial effects on recovery from AKI, it has also been demonstrated that formoterol accelerates podocyte recovery from glomerular injury by inducing MB (Arif et al., 2019), providing evidence that improving mitochondrial function has a beneficial therapeutic effect on multiple 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, all 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.

Authorship Contributions

K.H.C. and R.G.S were involved in the conception, design and interpretation of experiments and wrote the manuscript.

K.H.C. performed experiments and analyzed data.

Figure 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.

Figure 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.

Figure 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.

Figure 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.

Figure 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.

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Figure 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.

Figure 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.

Figure 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 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.

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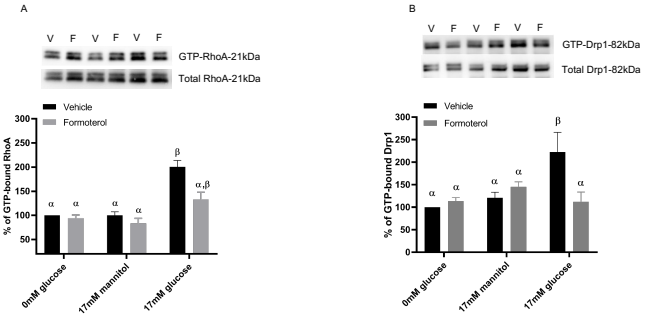
Figure 1



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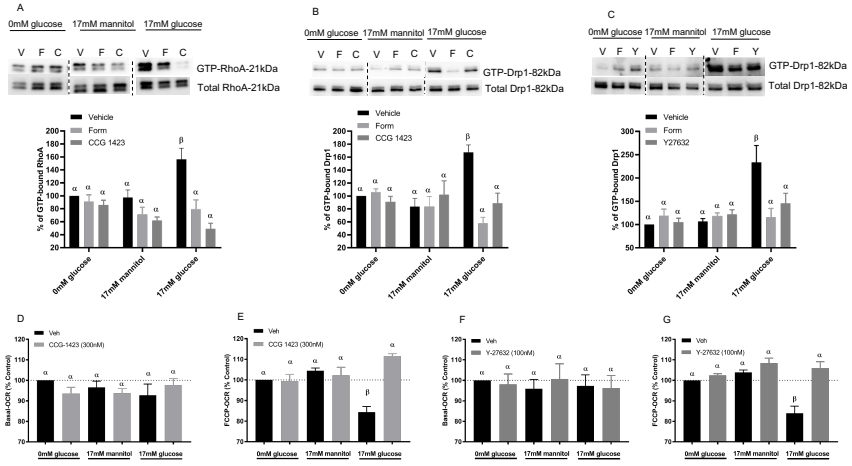

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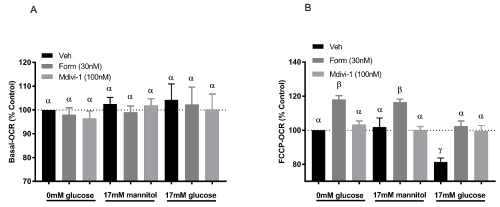

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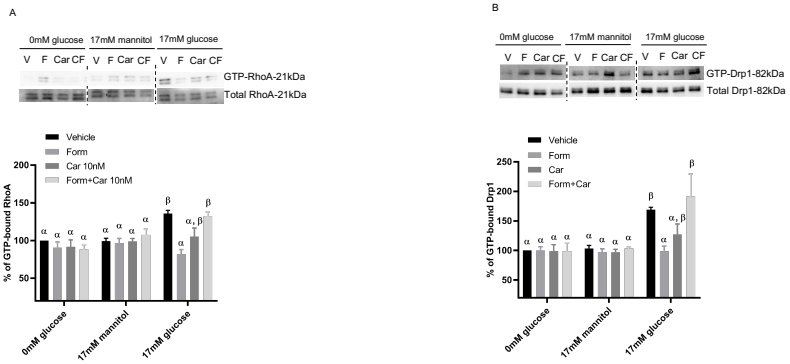
Figure 4



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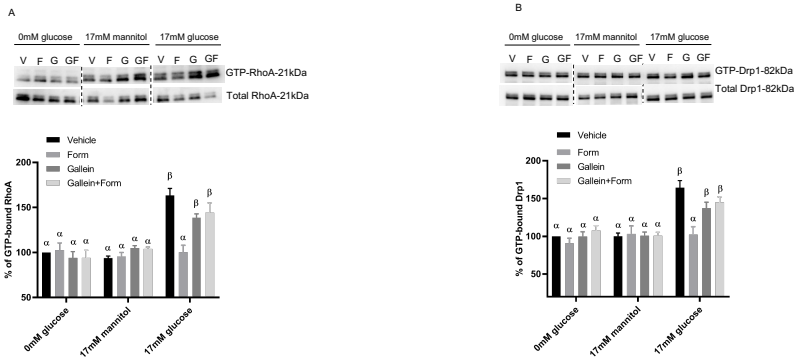

Figure 6

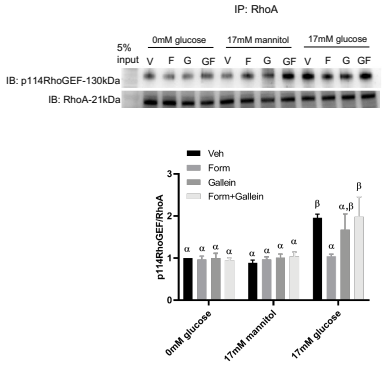

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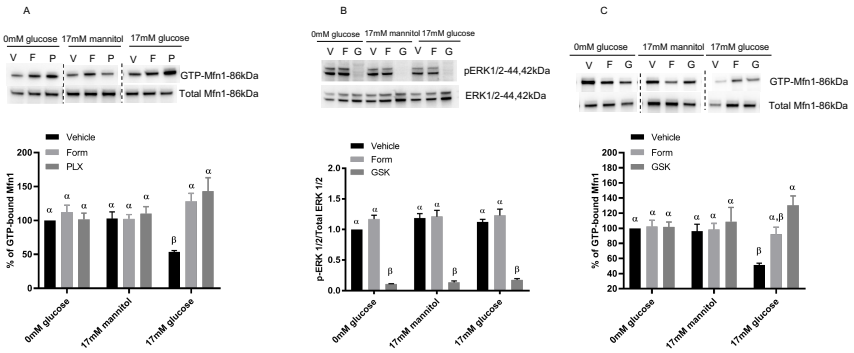
Figure 7



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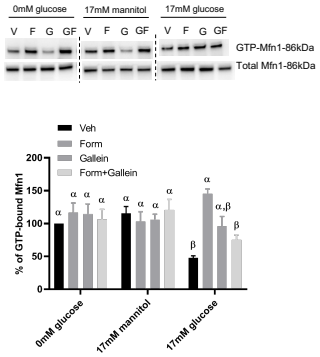

Figure 9