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The Role of Soluble Guanylyl Cyclase Signaling in Mitochondrial Biogenesis and Renal Injury

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By

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Dedication

This dissertation is dedicated to my loving family (Mom, Dad, Anchal, and Akash) who have experienced and gone through all the ups and downs in this journey with me and yet still managed to keep me standing tall. They are stronger than I am and I would not have made it to the end without them. Thank you for always reminding me who I am and what I am made of – Love, Pallu.

Acknowledgements

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List of Abbreviations

$5\text{-}HT_{1F}$: 5-hydroxytryptamine receptor 1F

8-Br-cGMP: 8-bromo-cyclic guanosine monophosphate

ADP: Adenosine Diphosphate

AGE: Advanced Glycation End Products

AICAR: 5-Aminoimidazole-4-carboxamide ribonucleotide

AKI: Acute Kidney Injury

AKIN: Acute Kidney Injury Network

AKT: Protein Kinase B

AMP: Adenosine Monophosphate

AMPK: 5' adenosine monophosphate-activated protein kinase

AQP1: Aquaporin 1

Arg1: Arginase 1

Atg7: Autophagy Related Protein 7

ATP: Adenosine Triphosphate

ATPase: adenosine triphosphatase

β_2AR: β_2-Adrenergic Receptor

Bak: Bcl-2 homologous antagonist/killer

Bax: Bcl-2-associated X protein

Bcl2: B-cell lymphoma 2

BNIP3: BCL2/adenovirus E1B 19 kDa protein-interacting protein 3

BUN: Blood Urea Nitrogen

CD36: platelet glycoprotein 4

cAMP: cyclic adenosine monophosphate

CaN: Calcinuerin

cGMP: cyclic guanosine monophosphate

CKD: Chronic Kidney Disease

CNGs: cGMP gated ion channels

CO_2: Carbon Dioxide

CoA: Coenzyme A

CPT1: Carnitine palmitoyltransferase I

CREB: cAMP Response element binding protein

Csf: Colony Stimulating Factor

DN: Diabetic Nephropathy

Drp1: Dynamin-Related protein 1

ENAC: Epithelial Sodium Channel

ERDF: Endothelial-Derived Relaxing Factor

ERK: Extracellular single-regulated kinase

ERRs: Estrogen-related receptors

ESRD: End Stage Renal Disease

ETC: Electron Transport Chain

$FADH_2$: Flavin adenine dinucleotide

FIS1:Mitochondrial Fission 1

FUNDC1: FUN14 domain-containing protein 1

GFR: Glomerular Filtration Rate

GKIPs: cGMP-dependent protein kinase-interacting proteins

GPCRs: G-protein coupled receptors

GPx: Glutathione Peroxidase

GSH: Glutathione

GSK3β: Glycogen Synthase

GSNO: S-Nitrosoglutathione

GSNOR: S-Nitrosoglutathione Reductase

GTP: Guanosine tri-phosphate

HFD: High Fat Diet

HRE: Hypoxia-Response Element

HIF-1α: Hypoxia-inducible factor 1-alpha

HIF-1β: Hypoxia-inducible factor 1-beta

HNOX: Heme-nitric oxide/oxygen binding

IHG-1: Induced in High Glucose-1

I/R: Ischemia and Reperfusion

IRI: Ischemia and Reperfusion Injury

KDIGO: Kidney Disease Improving Global Outcomes

KEGG: Kyoto Encyclopedia of Genes and Genomes

KIM1: Kidney Injury Marker-1

LC3: Microtubule-associated protein 1A/1B-light chain 3

MAPK: Mitogen-activated protein kinases

MB: Mitochondrial Biogenesis

MEKK: Mitogen-activated protein kinase kinase kinase

MFF: Mitochondrial Fission Factor

MFN: Mitofusin

mGSH: mitochondrial Glutathione

MID: Mitochondrial dynamics protein

MR: Mannose Receptor

Msr1: Macrophage Scavenger Receptor 1

mtDNA: mitochondrial DNA

mTOR: mammalian target of rapamycin

MTORC1: mammalian target of rapamycin complex 1

MTORC2: mammalian target of rapamycin complex 2

NADH: Nicotinamide adenine dinucleotide hydrogen

NADPH: nicotinamide adenine dinucleotide phosphate hydrogen

NGAL: Neutrophil gelatinase-associated lipocalin

NiPKO: nephron-specific inducible PGC-1α knockout

Nix: BCL2 Interacting Protein 3 Like

NKCC2: Na-K-Cl cotransporter

NRF2: nuclear factor erythroid 2–related factor 2

NOS: nitric oxide synthase

Oma1: mitochondrial Metalloendopeptidase

ONOO-: Peroxynitrate

OPA1: Optic Atrophy 1

PAH: Pulmonary Arterial Hypertension

PARKIN: ubiquitin ligase

PAS: Per-ARNT-Sim

PDEs: phosphodiesterases

PDE5: phosphodiesterase 5

PGAM5: serine/threonine-protein phosphatase

PGC-1α: Peroxisome proliferator-activated receptor-gamma coactivator 1α

PINK1: PTEN-induced kinase 1

PKA: Protein Kinase A

PKC: Protein Kinase C

PKG: Protein Kinase G

PKM2: Pyruvate kinase muscle isozyme M2

PPARs: peroxisome proliferator-activated receptors

PTH: Parathyroid hormone

RHEB: Ras homolog enriched in brain

RhoA: Ras homolog gene family, member A

RICTOR: Rapamycin-insensitive companion of mammalian target of rapamycin

RIFLE: risk, injury, failure, loss, and end-stage renal disease

ROCK1: Rho-associated protein kinase 1

ROS: Reactive Oxygen Species

RPTC: Renal Proximal Tubule Cells

sCr: Serum Creatinine

sGC: Soluble Guanylyl Cyclase

SIRT1: Sirtuin 1

SIRT3: Sirtuin 3

SOD2: Superoxide dismutase 2

TCA: Tricarboxylic Acid Cycle

UCP2: Mitochondrial uncoupling protein 2

ULK: Unc-51 like autophagy activating kinase

VASP: Vasodilator Stimulated Phosphoprotein

YME1L: ATP-dependent metalloprotease YME1L1

YY1: Yin Yang 1

Abstract

PALLAVI BHARGAVA. The Role of Soluble Guanylyl Cyclase Signaling in Mitochondrial Biogenesis and Renal Injury. (Under the direction of RICK G. SCHNELLMANN)

Soluble Guanylyl Cyclase (sGC) is responsible for converting GTP to cyclic GMP (cGMP). sGC function and regulation is complex and cell-type dependent. An increase in cGMP production can target phosphodiesterases (PDEs), cGMP gated ion channels, and protein kinase G (PKG). Increasing sGC activity and inhibiting the degradation of cGMP by targeting PDEs are two approaches to maintaining cGMP levels in a given system. Interestingly, cGMP can also regulate mitochondrial biogenesis (MB), the generation of new and functional mitochondria.

Previously in our laboratory, we have shown that MB is suppressed after ischemia and reperfusion (I/R) injury and certain mitochondrial biogenic compounds can accelerate recovery and attenuate the decrease in MB. In particular, we have shown that administration of an inhibitor of PDE5, sildenafil, can restore MB and renal function after I/R. However, the role of sGC in I/R is still under investigation. Here, we have elucidated a potential signaling pathway for the involvement of sGC in the suppression of MB. We performed I/R on mice and focused on the events that occur within the first 24 h of I/R injury. We optimized an sGC enzyme assay for the kidney to better understand the redox state of sGC. We proposed that the suppression of MB is cGMP-dependent and that PKG is a mediator of these effects. Moreover, we have proposed a role for ERK1/2 in the sGC/cGMP/PKG induced suppression of MB.

Alternatively, we have shown that cGMP can induce MB in renal proximal tubule cells by 24 h with exposure to a cGMP analog, 8-Br-cGMP. However, the mechanism behind this finding is unknown. Therefore, we treated renal proximal tubule cells with a 8-Br-cGMP, for an 1 h to elucidate the mechanism behind this event. We measured the phosphorylation of serine and threonine residues on PGC-1α, the master regulator of MB, since phosphorylation can prolong the half-life of PGC-1α and induce MB. We found that the induction of MB is PKG dependent and that p38 MAPK plays a prominent role in the phosphorylation of PGC-1α.

Here, we present sufficient evidence for the role of sGC signaling in regulating MB in the kidney.

Chapter 1: Introduction to the Role of Soluble Guanylyl Cyclase Signaling in Mitochondrial Biogenesis and Renal Injury

Renal Anatomy

The kidney is one of the most energy-demanding organs in the human body. A study measuring the resting energy expenditure of various organs in healthy adults, ranging from 21 to 73 years of age, found that the kidney and heart had the highest resting metabolic rates[1]. The kidney has the second highest mitochondrial content and oxygen consumption after the heart[2,3]. The resting metabolic rate for the kidney is high because the kidney requires an abundance of mitochondria to provide sufficient energy to enable it to remove waste from the blood, reabsorb nutrients, regulate the balance of electrolytes and fluid, maintain acid-base homeostasis, and regulate blood pressure.

Blood is first received from the afferent arteriole and filtered through the glomerulus where glucose, ions, urea, and water are able to pass and larger molecules such as hemoglobin are retained. Proximal tubules reabsorb 80% of the filtrate that passes through the glomerulus, including glucose, ions, and nutrients. These tasks, especially the reabsorption of glucose, ions and nutrients through channels and transporters, are driven by ion gradients. Filtrate then passes through the loop of Henle consisting of the thin descending limb, thin ascending limb, and thick ascending limb. The thin descending limb reabsorbs water via passive transport whereas the thin ascending limb is impermeable to water reabsorption. Tight junctions in the thin descending limb are considered to be more leaky than tight junction in the thin ascending limb. Ion reabsorption continues in the thin ascending limb and thick ascending limb however, the process in the thick ascending limb requires active transport. Sodium reabsorption occurs

in the thick ascending limb through membrane transport proteins, NKCC2, that are located on the apical membrane of the thick ascending limb. NKCC2 transport 2 Cl-, 1 K+, and, 1 Na+ from the urine across the membrane into the thick ascending limb. Subsequently, Na+K+ATPases that are located in the basolateral membrane of the thick ascending limb, between the bloodstream and thick ascending limb, pump 3 Na+ ions in the blood and 2 K+ ions into the thick ascending limb. This allows for the inside of the thick ascending limb to be negatively charged and therefore generates an electrochemical gradient for proper movement of ions across NKCC2. Filtrate from the loop of Henle is further filtered in the distal convoluted tubule reabsorbing Ca2+, K+, Na+ ions. In order to maintain optimum pH, bicarbonate and H+ ions can be reabsorbed or secreted in the filtrate. Hormones such as aldosterone and parathyroid hormone (PTH) exhibit their effects in the distal convoluted tubule and collecting ducts. Aldosterone can cause the reabsorption of Na+ ions and the secretion of K+ ions into the filtrate creating downstream effects such as changes in blood pressure, blood volume, and water retention. PTH is secreted to cause the retention of Ca^{2+} in the kidney, bone, and intestine. In terms of the kidney, PTH exhibits its effects in the proximal tubule, distal convoluted tubule, and collecting ducts. The resulting filtrate is retained in the collecting duct until excreted into the renal pelvis. The process of filtration as explained, involves active and passive transport mechanisms that allow for the excretion of wastes and retention of nutrients, ions, and glucose.

As mentioned above, Na+,K+-ATPases play a large role in the active transport mechanisms used to allow for proper filtration of blood in the kidney. Mitochondria

provide energy to Na^+,K^+-ATPase to generate ion gradients across the cellular membrane[4]. In the kidney the proximal tubule, the loop of Henle, the distal tubule and the collecting duct all require active transport to reabsorb ions[4]. This is in contrast to glomerular filtration, which is a passive process that is dependent on maintaining hydrostatic pressure in the glomeruli. Proximal tubules contain the most ATP-dependent ion transporters in the kidney since that reabsorb 80% of the filtrate and as such, they contain more mitochondria than any other structure in the kidney. The ability of mitochondria to sense and respond to changes in nutrient availability and energy demand by maintaining mitochondrial homeostasis is critical to the proper functioning of the proximal tubule.

Mitochondrial Biology

Mitochondria are a network of plastic organelles that together maintain a variety of cellular functions and processes, such as the level of reactive oxygen species, cytosolic calcium and apoptosis[5]. Most importantly, mitochondria produce ATP, thereby supplying the energy source for basal cell functions as well as cellular repair and regeneration. To accomplish this feat, a population of healthy and functional mitochondria is vital.

[H2] ATP production

Aerobic respiration is the consumption of oxygen to produce ATP, water and carbon dioxide. Most of the ATP generated by aerobic respiration is produced by the flux of electrons through the electron transport chain (ETC) in a process called oxidative

phosphorylation (FIG. 1.1a). Aerobic respiration begins with the production of pyruvate from glucose via glycolysis[6]. Pyruvate is converted to acetyl CoA (via pyruvate dehydrogenase in the mitochondrial matrix), which fuels the tricarboxylic acid (TCA) cycle to produce six reduced nicotinamide adenine dinucleotide (NADH), four flavin adenine dinucleotide ($FADH_2$), and six CO_2 per molecule of glucose[6]. Electrons from NADH and $FADH_2$ are transferred to complex I and complex II, respectively, of the ETC in the mitochondrial inner membrane. Electrons travel through the ETC to complex IV, in which they are accepted by oxygen. Note that the haemeprotein Cytochrome c, which is located in the mitochondrial inner membrane, facilitates the transfer of electrons from complex III to complex IV. Ultimately protons, which are actively pumped into the intermembrane space as electrons move through complexes I, III, and IV, flow through ATP synthase (also known as complex V) to drive the conversion of ADP to ATP[6].

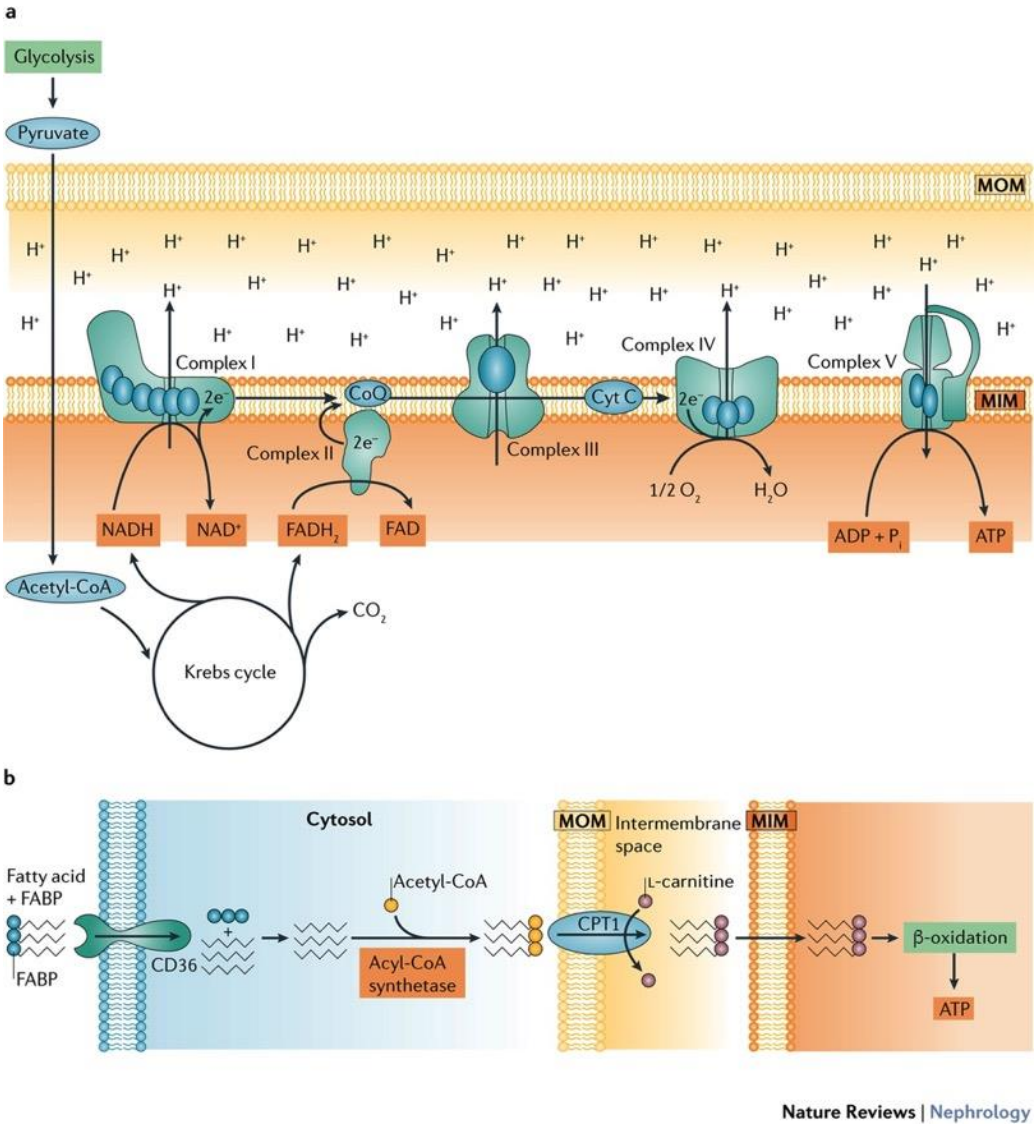

Figure 1.1: ATP production in the kidney

The electron transport chain (ETC). A functioning ETC transforms reducing equivalents from NADH and $FADH_2$ to produce NAD^+ and FAD^+, respectively. The electrons (e^-)that are produced travel through the complexes of the ETC and are ultimately accepted by oxygen at complex IV. As electrons are transferred from complex to complex, protons (H^+) are actively pumped out from complexes I, III, and IV into the intermembrane space, maintaining the membrane potential and driving the production of ATP by ATP synthase (also known as complex V). **b** | Fatty acid transport and activation in renal proximal tubule cells. Proximal tubules require large amounts of ATP to drive ion transport and therefore rely on aerobic respiration, the most efficient mechanism for producing ATP. Fatty acids are a main source of energy for proximal tubules because more ATP can be produced from one molecule of palmitate than from one molecule of glucose[18]. Fatty acids bound to fatty acid-binding proteins (FABP) are transported into the proximal tubule cell via platelet glycoprotein 4 (also known as CD36) and activated by the addition of acetyl-CoA in the cytosol via acyl-CoA synthetase. Activated fatty acids are transported into mitochondria via carnitine *O*-palmitoyltransferase 1 (CPT1), which exchanges their acyl-CoA group for L-carnitine, whereupon they undergo β-oxidation to produce ATP. CoQ, coenzyme Q; Cyt C, cytochrome *c*; MIM, mitochondrial inner membrane; MOM, mitochondrial outer membrane; P_i, inorganic phosphate.

From: Bhargava, P. & Schnellmann, R. G. (2017) Mitochondrial energetics in the kidney
Nat. Rev. Nephrol. doi:10.1038/nrneph.2017.107

In general, all cell types in the kidney need ATP to maintain cellular functions; however, the mechanism by which ATP is produced is cell type dependent. For example, in the renal cortex, proximal tubules depend on the efficiency of oxidative phosphorylation to produce ATP that drives active transport of glucose, ions, and nutrients.[7] In contrast, glomerular cells including podocytes, endothelial cells, and mesengial cells have lower oxidative capacity because their function is to filter blood, removing small molecules (glucose, urea, water, and salts) while retaining large proteins such as hemoglobin.[8] This passive process does not directly require ATP and therefore glomerular cells have the ability to perform aerobic and anaerobic respiration to produce ATP for basal cell processes.[9-12] Anaerobic respiration also begins with glycolysis like aerobic respiration, producing pyruvate from glucose, but is characterized by the production of lactate from pyruvate[13]. This is important as cell types other than proximal tubules perform glycolysis more often and are able to utilize other energy sources such as amino acids in the absence of glucose[14,15]. For example, pyruvate can be generated via the oxidation of amino acids fueling both anaerobic and aerobic mechanisms for ATP production.

Due to the high energy demand of proximal tubules, aerobic respiration is the primary mechanism for ATP production. Proximal tubules utilize non-esterified fatty acids, such as palmitate, via β-oxidation for maximal ATP production. A single molecule of palmitate produces 106 molecules of ATP whereas the oxidation of glucose only yields 36 molecules of ATP[16,17]. Fatty acids are taken up by proximal tubule cells via transport proteins, such as platelet glycoprotein 4 (also known as CD36), or synthesized in the cytoplasm where they are activated by coenzyme A prior to transport into mitochondria

through the carnitine shuttle (FIG. 1.1b)[18]. Specifically, carnitine O-palmitoyltransferase 1 (CPT1) exchanges the coenzyme A group on fatty acids with L-carnitine, allowing the transfer of fatty acids across the mitochondrial inner membrane space through the carnitine shuttle. Fatty acids are then broken down for energy via β-oxidation in the mitochondrial matrix. While β-oxidation is the most efficient mechanism for maximal ATP production for proximal tubules, it is important to note that due to the high consumption of oxygen by proximal tubules, they are more susceptible to changes in oxygen levels.[19,20] A decrease in oxygen levels can ultimately lead to the impairment of β-oxidation and the reduction of ATP production (discussed below).

A balance of catabolic and anabolic nutrient sensing pathways regulates the optimal concentration of fatty acids in a cell (see below). Disease states and different metabolic conditions in the kidney alter this balance and can adversely affect mitochondrial energetics. For example, the accumulation of fatty acids in AKI and DN can negatively impact ATP production by decreasing β-oxidation in the mitochondria and increasing the formation of lipid droplets inside the cell[17]. An inverse correlation exists between lipogenesis that is induced by the accumulation of fatty acids and the transcription of genes, the protein products of which are involved in fatty acid oxidation[21,22]. Fatty acids can also trigger apoptosis and more importantly, create a toxic environment inside the cell that hinders mitochondrial function[23,24]. Fatty acid metabolism in disease states such as AKI and DN will be discussed below.

[H2] Antioxidant Defenses

As discussed, mitochondria produce ATP via the ETC. At steady state, when electrons are passed through the ETC to molecular oxygen, - a low concentration of superoxide anions are generated from complex I and complex III. Although a low level of reactive oxygen species (ROS) such as these is important for cell function, high concentrations are toxic to mitochondria and the cell (FIG. 1.2)[25-27]. For example, under oxidative stress, increased levels of ROS can cause breaks in mitochondrial DNA (mtDNA) that cause mutations in the next generation of mitochondria, negatively affecting the efficiency of the ETC causing a decrease in ATP production, and damage proteins and lipids[28]. ROS can also trigger apoptosis in the cell by causing the release of cytochrome c and leading to mitochondrial dysfunction[28]. Therefore, mitochondria have antioxidant defense systems to counteract the excessive formation of additional ROS. Superoxide dismutase 2 (SOD2), which converts superoxide anions to hydrogen peroxide and oxygen, is specific for mitochondria[29]. Moreover, the transcription of genes encoding antioxidant enzymes such as SOD2, catalase, and glutathione peroxidase is activated by nuclear factor erythroid 2-related factor 2 (NRF2), in response to oxidative stress, providing a mechanism to prevent excessive ROS production [30]. The importance of these antioxidant systems is to maintain optimal ATP production and sustain mitochondrial function.

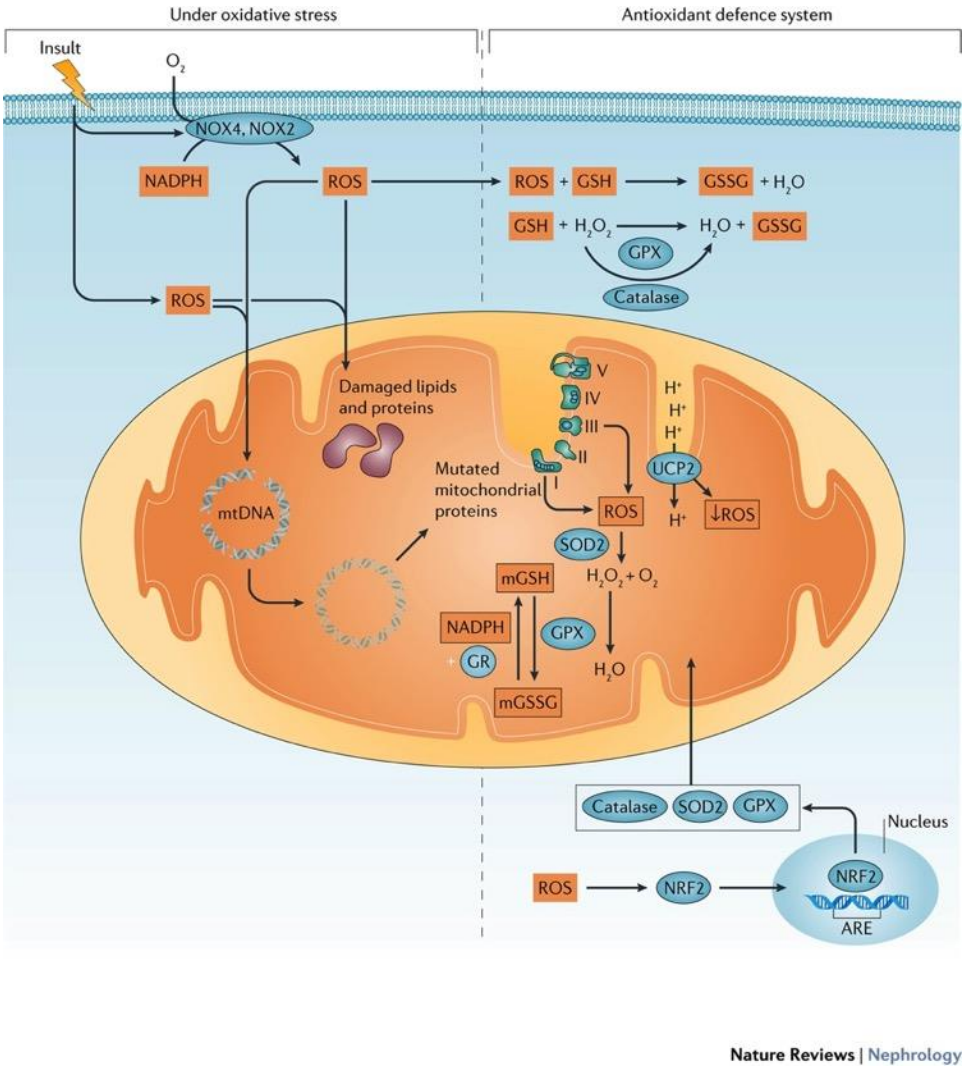

Figure 1.2: Oxidative Stress and the Antioxidant Defence System
Insults can increase the production of reactive oxygen species (ROS) in the cytosol and mitochondria. NADPH oxidase 2 (NOX2) and NOX4 can also contribute to the production of ROS[222]. The production of ROS can cause breaks in mitochondrial DNA (mtDNA) and damage lipids and proteins. Damaged mtDNA can produce aberrant mitochondrial proteins and prevent mitochondrial protein synthesis, whereas damaged lipids and proteins result in impaired mitochondrial function, leading to further increases in mitochondrial ROS. ROS also activate nuclear factor erythroid 2-related factor 2 (NRF2), which translocates to the nucleus and binds to antioxidant-responsive elements (AREs) to activate the transcription of genes encoding oxidant-neutralizing enzymes, such as mitochondrial superoxide dismutase 2 (SOD2), glutathione peroxidase (GPX) and catalase. SOD2 reduces superoxide anions to hydrogen peroxide (H_2O_2) and oxygen (O_2). Catalase, found in the cytoplasm, and GPX, located in the cytoplasm and mitochondria, reduce H_2O_2 to water (H_2O)[223]. GPX also oxidizes glutathione (GSH), resulting in glutathione disulfide (GSSG) as a byproduct of reducing hydrogen peroxide to water. GSSG in mitochondria (mGSSG) is converted back to GSH by glutathione reductase (GR) in a process that requires the presence of NADPH. The activity of the mitochondrial uncoupling protein 2 (UCP2) is increased, dissipating the proton motive force and decreasing ROS production. mGSH, mitochondrial GSH. The electron transport chain complexes I–V are indicated as I, II, III, IV and V. From: Bhargava, P. & Schnellmann, R. G. (2017) Mitochondrial energetics in the kidney *Nat. Rev. Nephrol.* doi:10.1038/nrneph.2017.107

Another important antioxidant defense mechanism involves glutathione. Glutathione is a tripeptide (γ-glutamyl-cysteinal-glycine) nucleophile that can exist in a reduced form (GSH), or in the oxidized form as glutathione disulfide (GSSG). Mitochondria contain their own pool of glutathione, mGSH, which not only helps to decrease excessive ROS levels but also to prevent the release of cytochrome c from the inner membrane. mGSH directly interacts with superoxide anions and becomes oxidized to GSSG[31]. Glutathione peroxidase (GPx) is located in both the cytoplasm and the mitochondria, and uses GSH to reduce hydrogen peroxide to water, resulting in GSSG as a by-product[32] . GSSG cannot exit the mitochondria and is converted back to mGSH, by glutathione reductase, for use again or eliminated from the mitochondria[31]. The conversion of GSSG to mGSH requires nicotinamide adenine dinucleotide phosphate (NADPH), allowing crosstalk between the mechanism that maintains mGSH levels and the pentose phosphate pathway that produces NADPH. Taken together, these mechanisms play a major role in preventing excessive levels of ROS to sustain mitochondrial function.

Uncoupling proteins are a family of mitochondrial transport proteins located in the mitochondrial inner membrane[33,34]. They transport protons across the inner membrane to the mitochondrial matrix. Mitochondrial uncoupling protein 2 (UCP2) is expressed in the kidney and it is activated by mitochondrial ROS and other stimuli. An increase in ROS formation in the mitochondria activates UCP2, dissipating the proton motive force as heat and, as a result, reducing ROS production[34,35]. As ROS production contributes to mitochondrial dysfunction in AKI and diabetic nephropathy, UCP2 has been explored in the kidney and in these disease states[36]. Studies investigating the role of *UCP2* gene

polymorphisms in the kidney that exacerbate disease in patients with diabetic nephropathy reveal that UCP2 is a potential target for treatment[37]. It was also reported that a lack of UCP2 worsened tubular injury after AKI in mice[36]. These studies show the importance of UCP2 in the kidney as well as its role in attenuating excessive ROS production.

There are also mechanisms that sustain mitochondrial function under hypoxic conditions. The lack of oxygen under hypoxic conditions decreases ATP production and causes cell death. In normoxic conditions, hypoxia-inducible factor-1α (HIF-1α) is degraded in the presence of oxygen and α-ketoglutarate, an intermediate of the TCA cycle[38]. However, under hypoxic conditions HIF-1α heterodimerizes with HIF-1β to form a transcription factor that binds to a hypoxia response element (HRE) that is present in genes encoding glycolytic enzymes and glucose transporters in the kidney[39]. Hypoxic conditions also alter the composition of complex IV of the ETC in which, at physiological conditions, regulatory subunit 1 predominates in the ETC; during hypoxia regulatory subunit 2 predominates in Complex IV, which increases the efficiency of the ETC[40]. Several studies have shown that increasing the efficiency of the ETC increases the production of mitochondrial ROS under hypoxic conditions, although this mechanism is still unclear[41-43]. The effects of oxidative stress and hypoxia on mitochondrial morphology and energetics are discussed below.

Maintaining mitochondrial homeostasis

Nutrient-sensing pathways in the kidney

Nutrient-sensing pathways can directly affect mitochondrial energetics in response to external stimuli such as hypoxia, oxidative stress and energy depletion. Two signalling pathways in particular have been extensively explored in the kidney, namely the mechanistic target of rapamycin (mTOR) and AMP-activated protein kinase (AMPK) signalling pathways[44,45]. Both signalling pathways also have a role in regulating mitochondrial biogenesis (MB) — that is, the production of new and functional mitochondria — to help maintain a healthy population of mitochondria (FIG. 1.3).

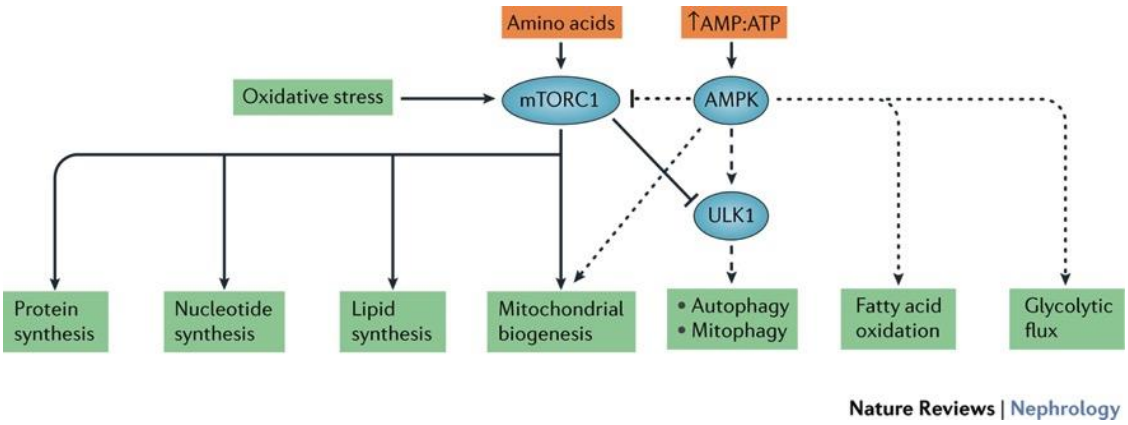

Figure 1.3: Crosstalk between two nutrient-sensing pathways
Mechanistic target of rapamycin complex 1 (mTORC1) and AMP-activated protein kinase (AMPK) have key roles in regulating mitochondrial biogenesis and mitophagy. mTORC1 is responsible for triggering anabolic pathways, such as the synthesis of proteins, nucleotides and lipids, as well as mitochondrial biogenesis. AMPK activates catabolic pathways, including autophagy, mitophagy, fatty acid oxidation and glycolysis. AMPK can stimulate mitochondrial biogenesis (dotted arrow). However, in response to stimuli such as nutrient deprivation, AMPK can inhibit mTORC1 (dotted inhibitory line) and phosphorylate ULK1 to activate mitophagy (dashed arrow). Together these two signalling pathways maintain cell function and sustain mitochondrial energetics in response to stimuli such as hypoxia, oxidative stress and energy depletion. From: Bhargava, P. & Schnellmann, R. G. (2017) Mitochondrial energetics in the kidney *Nat. Rev. Nephrol.* doi:10.1038/nrneph.2017.107

mTOR is a serine/threonine kinase that is made up of a complex of proteins. There are two distinct complexes of mTOR, mechanistic target of rapamycin complex 1 (mTORC1) and mTORC2, each of which contain their own unique subunits and substrates. mTORC1, a complex of mTOR, regulatory-associated protein of mTOR (Raptor) and several other proteins, regulates cell growth and proliferation and inhibits autophagy by stimulating anabolic processes. mTORC2, which is a complex of mTOR, rapamycin-insensitive companion of mTOR (Rictor) and several other proteins, is thought to regulate potassium and sodium levels in the kidney[46,47]. mTORC1 is considered a nutrient sensor because it can be activated by growth factors, nutrients such as amino acids and glucose, and oxidative stress, triggering pathways that lead to protein synthesis, nucleotide synthesis, lipid synthesis, and MB by activating the transcriptional repressor yin-yang 1, (YY-1)[44,48]. In the case of MB, YY-1 acts as a transcription factor and coactivator of the master regulator of MB- the transcriptional coactivator peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) - resulting in the transcription of mitochondrial genes.[48] mTORC1-decficiency specifically in renal proximal tubules of mice decreased the protein levels of PGC-1α , *in vivo*.[49] Of note, the mTOR pathway can be inhibited by hypoxia and AMPK.

AMPK is another nutrient sensor in the kidney that stimulates catabolic processes. When the AMP:ATP ratio in the cell is high in the presence of low oxygen levels, AMPK is activated. AMPK targets a number of proteins, the phosphorylation of which leads to the production of antioxidant enzymes, the induction of MB, and an increase in glycolytic flux, fatty acid oxidation and glucose transport; all of these events contribute to cell

growth and an increase in cellular metabolism[50]. AMPK can induce MB by stimulating the transcription of the gene encoding PGC-1α and by phosphorylating PGC-1α at threonine 177 and serine 539 to increase its activity[51]. AMPK stimulates the production of energy and inhibits energy-consuming pathways by inhibiting mTORC1. Under conditions of nutrient deprivation, crosstalk exists between mTORC1 and AMPK (FIG. 3) so that AMPK can inhibit mTORC1 while activating autophagy by phosphorylating the serine/threonine-protein kinase ULK1[52]. Due to its high number of targets in kidney cells, AMPK is a novel drug target for several diseases in the kidney (see below).

Mitochondrial homeostasis is a balance between MB, fission and fusion, and mitophagy, the selective removal of non-functional and damaged mitochondria from cells by autophagy. All of these processes work in concert to maintain mitochondrial energetics, that is, the optimal production of ATP in normoxic conditions and in altered metabolic condition.

Mitochondrial Biogenesis

Mitochondrial biogenesis (MB), which produces new and functional mitochondria, increases ATP production in response to increasing energy demands. MB is regulated by an array of transcriptional coactivators and corepressors[53,54]. PGC-1α. was shown to be a prominent regulator, at the transcriptional level, of oxidative phosphorylation, the TCA cycle and fatty acid metabolism in the kidney[55]. The investigators performed gene expression profiling of kidneys from control mice and nephron-specific inducible PGC-

1α knockout (NiPKO) mice that had been fed a chow diet or high fat diet (HFD). Using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database,[link to: http://www.genome.jp/kegg/] they analyzed transcripts from all four groups of mice. Interestingly, a decrease in transcripts related to oxidative phosphorylation, TCA cycle, and glycolysis was detected in chow-fed NiPKO mice and in HFD-fed NiPKO mice. This finding supports the idea that inactivation of PGC-1α in the kidney significantly reduces mitochondrial function and metabolism and subsequently decreases MB.

It has also been shown that the overexpression of PGC-1α can mitigate mitochondrial dysfunction *in vitro* after oxidant exposure, further supporting a role for MB in mitochondrial homeostasis[56]. The activation of peroxisome proliferator-activated receptors (PPARs) and estrogen-related receptors (ERRs) also contributes to the regulation of MB, sometimes by directly interacting with PGC-1α[57](FIG. 1.4). PPARs and ERRs are nuclear receptors that can be activated by fatty acids and steroid hormones such as estrogen, and they elicit a response by binding to specific DNA response elements through their DNA-binding domains[58]. PGC-1α can directly bind to these nuclear receptors and coactivate the transcription of genes for oxidative phosphorylation and fatty acid oxidation[59,60]. PGC-1α activation results in its translocation from the cytoplasm to the nucleus, allowing it to upregulate the transcription of genes, the protein products of which are important for mitochondrial homeostasis and ATP production[61]. Transcription programmes downstream of PGC-1α include nuclear and mitochondrial genes, as well as those involved in signalling pathways that regulate MB (reviewed elsewhere)[62-64].

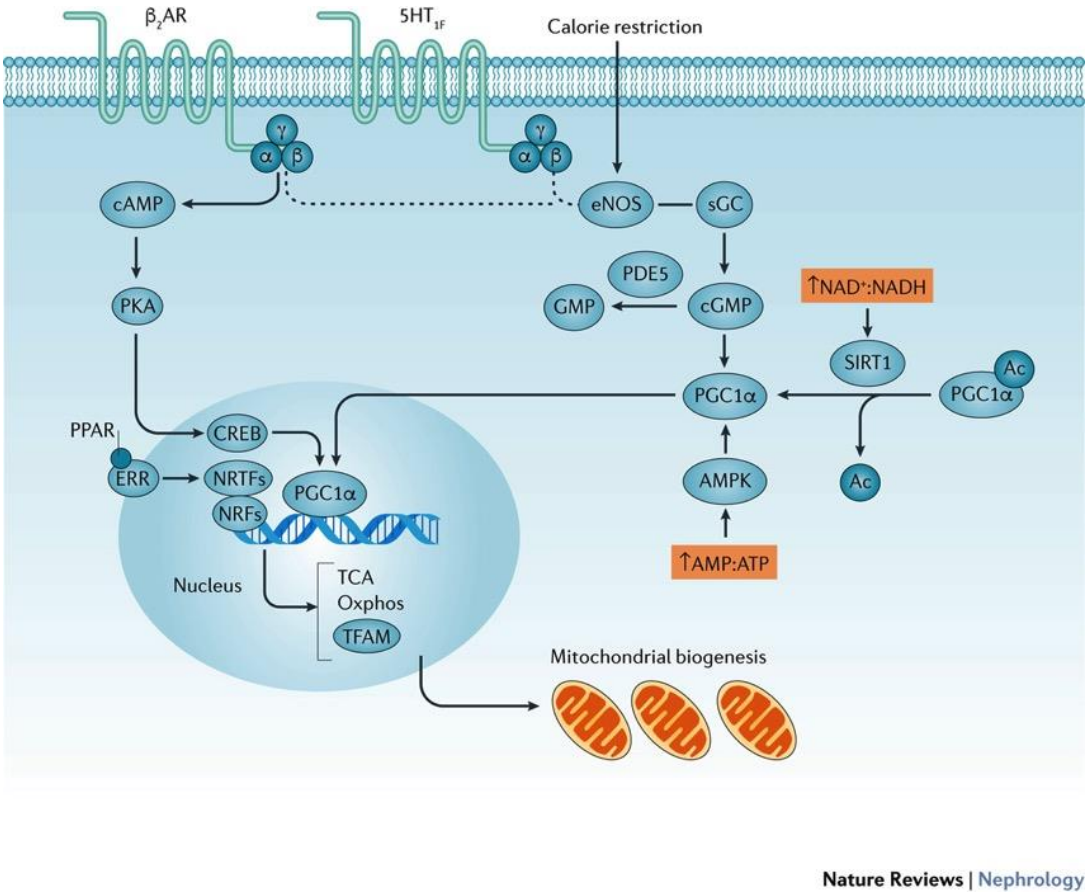

Figure 1.4: A complex network of pathways regulate mitochondrial biogenesis. Activation of peroxisome proliferator-activated receptor-γ co-activator 1α (PGC1α) in the cytosol causes its translocation to the nucleus and the transcription of genes (including that encoding mitochondrial transcription factor A (TFAM)), the protein products of which are needed for oxidative phosphorylation (oxphos), the tricarboxylic acid (TCA) cycle and mitochondrial biogenesis. TFAM aids in the transcription of genes that are encoded by mitochondrial DNA[224,225,226]. The activation of G protein-coupled receptors (GPCRs), such as the $\beta_2$adrenergic receptors (β_2AR) and 5-hydroxytryptamine receptor 1F (5-HT_{1F}), leads to the dissociation of heterotrimeric G proteins composed of Gα, Gβ and Gγ subunits and the subsequent activation of protein kinase A and endothelial nitric oxide synthase (eNOS)[66]. The pathway from GPCRs to eNOS is still under investigation, as indicated by the dashed line. eNOS stimulates soluble guanylyl cyclase (sGC) to form cyclic guanosine monophosphate (cGMP), which in turn activates PGC1α. A number of compounds can activate nuclear receptors such as peroxisome proliferator-activated receptors (PPARs) and oestrogen- related receptors (ERRs) and induce mitochondrial biogenesis. Once activated, these nuclear receptors can act as transcriptional co-activators (labelled in the figure as nuclear receptor transcription factors (NRTFs)), with PGC1α to stimulate mitochondrial biogenesis. Other transcription factors, including nuclear respiratory factor 1 (NRF1) and NRF2, can also directly bind to PGC1α to induce mitochondrial biogenesis[227]. Stimuli, such as caloric restriction, can activate eNOS, increasing the production of cGMP and leading to the activation of PGC1α. The activity of sirtuin 1 (SIRT1) is increased in the presence of a high ratio of NAD^+ to NADH concentrations, leading to the activation of PGC1α. High AMP:ATP ratios also activate AMP-activated protein kinase (AMPK), activating PGC1α by phosphorylation. In all of these cases, the activation of PGC1α stimulates mitochondrial biogenesis. Ac, acetyl; PDE5, cGMP-specific 3′,5′-cyclic phosphodiesterase; PKA, protein kinase A; sGC, soluble guanylyl cyclase. From: Bhargava, P. & Schnellmann, R. G. (2017) Mitochondrial energetics in the kidney *Nat. Rev. Nephrol.* doi:10.1038/nrneph.2017.107

As the activation or suppression of PGC-1α is regulated by external stimuli and post-translational modifications it can be considered to be a nutrient sensor in the kidney. PGC-1α protein expression in the kidney and its role is still being explored. However, much of what is known has been discovered in diseased states in the kidney caused by different insults such as DN, ischaemia/reperfusion injury (IRI), sepsis, cisplatin-induced AKI, and others. Findings in the diseased states support a role for the importance of PGC-1α in the recovery phase from these diseases and for restoring mitochondrial function, highlighting PGC-1α as a therapeutic target. Exercise and insulin stimulates an increase in PGC-1α gene expression in skeletal muscle and in the heart, whereas fasting will increase PGC-1α gene expression in the liver [62,65]. In brown fat and muscle cells, cold exposure activates PGC-1α [62]. In cases of oxidative stress or nutrient depletion, the activation of MB helps rescue mitochondria from apoptosis[66,67]. In general, if the cell is in need of more energy PGC-1α is activated by deacetylation; it is inactivated by acetylation when energy levels are high[62].

In addition to AMPK and mTOR, other energy sensing pathways that stimulate MB include those involving sirtuins, cyclic adenosine monophosphate (cAMP), and cyclic guanosine monophosphate (cGMP) (FIG. 1.4). NAD-dependent protein deacetylase sirtuin-1 (also known as SIRT1) and SIRT3 are protein deacetylases that play a role in a variety of mitochondrial processes, including the ETC, TCA cycle, fatty acid oxidation, redox homeostasis, and MB [68]. SIRT1 is activated by NAD+, and it can then activate downstream targets such as PGC-1α[61]. SIRT3 is a mitochondria-specific sirtuin and it can

be activated to stimulate MB [69]. The stimulation of adenylate cyclase results in an increase in cAMP, which activates protein kinase A (PKA) and, in turn, phosphorylates cyclic AMP-responsive element-binding protein (CREB)[62,70]. CREB is also a transcriptional activator of PGC-1α and therefore can also stimulate MB. Finally, increased levels of cGMP induced by caloric restriction and the inhibition of phosphodiesterase can stimulate PGC-1α activation and MB *in vivo*[71-73]. Several of these pathways are being targeted to increase MB to correct mitochondrial defects.

Mitochondrial dynamics and energetics

Mitochondrial morphology must be retained for maximal ATP production. The processes of fission, fusion and mitophagy drive mitochondrial dynamics as they directly impact mitochondrial structure and morphology. Fission and fusion complement each other under different metabolic conditions to maintain mitochondrial morphology, and mitophagy removes damaged mitochondria from the network[74]. Sustaining mitochondrial dynamics is important for retaining mitochondrial energetics.

[H3] Fission and fusion.

Fission, the splitting of mitochondria into two, and fusion, the combining of two mitochondria, are complementary processes necessary for mitochondrial homeostasis. At steady state there is a balance between these processes (FIG. 1.5). The genetic deletion of

genes, the protein products of which are involved in fission or fusion, causes human disease. For example, dominant optic atrophy is characterized by loss of visual acuity due to mutations in the gene encoding the fusion protein dynamin-like 120 kDa protein (also known as OPA1), and mutations in the gene encoding the fission protein dynamin-1-like protein (DRP1) are lethal [75-80]. Although there are exceptions, in general, studies have shown that there is an increase in oxidative phosphorylation in fusion and a decrease in oxidative phosphorylation in fission to match the energy demands of the cells[81,82]. Excessive fusion, like excessive fission, can also be associated with diseased states as seen in neurodegenerative diseases[83]. There are certain cell types that do not adhere to this trend such as adult cardiomyocytes and senescent cells. Mitochondria in adult cardiomyocytes have fragmented morphology and still maintain oxidative capacity whereas mitochondria in senescent cells remain elongated, characteristic of increased fusion[84]. Senescent cells in this elongated state show decreased bioenergetic capacity[85,86].

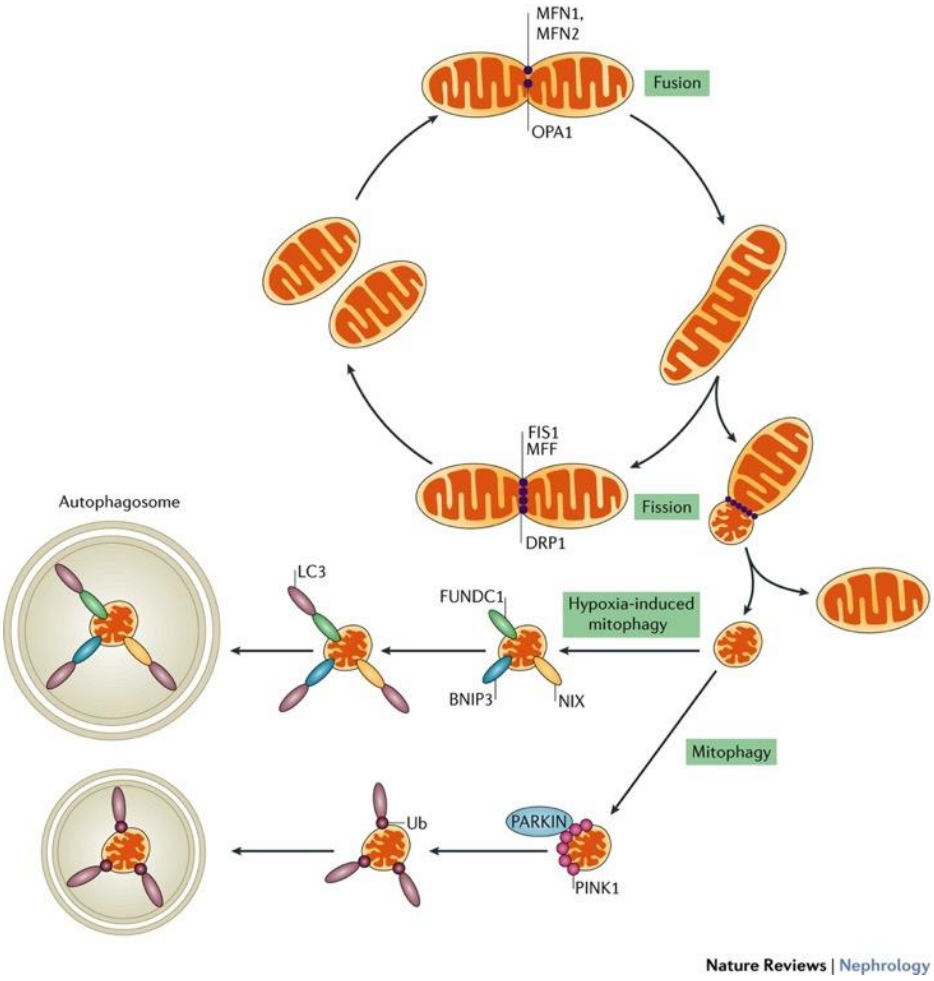

Figure 1.5: Mitochondrial dynamics: fission, fusion and mitophagy
Mitochondria are dynamic organelles that need to maintain their morphology for the optimal production of ATP under different metabolic conditions and as part of a healthy network of mitochondria. Fission and fusion are two processes that are necessary for the maintenance of mitochondria morphology. Mitochondria fuse together via mitofusin 1 (MFN1) and MFN2 (outer membrane fusion) and the activation of dynamin-like 120 kDa (OPA1) (inner membrane fusion). Fusion can occur to maintain ATP production or to redistribute mitochondrial proteins. Fission can isolate depolarized mitochondrion that might not contribute to the healthy network of mitochondria. The activation of fission causes the oligomerization of dynamin 1-like protein (DRP1) on the mitochondrial outer membrane, where it is bound to receptors (namely mitochondrial fission 1 (FIS1) and mitochondrial fission factor (MFF)), forming a ring-like structure that mediates the separation of mitochondria. The network also isolates dysfunctional mitochondria for degradation by mitophagy via a well-studied PTEN-induced putative kinase 1 (PINK1)–PARKIN mechanism. Under adverse conditions such as hypoxia, however, mitochondria will be removed by a FUN14 domain-containing protein 1 (FUNDC1) or BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3) and NIP3-like protein (NIX)-dependent mechanism of mitophagy. LC3, microtubule-associated protein 1 light chain 3; Ub, ubiquitin. From: Bhargava, P. & Schnellmann, R. G. (2017) Mitochondrial energetics in the kidney *Nat. Rev. Nephrol.* doi:10.1038/nrneph.2017.107

Fusion is a two-part process that involves fusing the outer mitochondrial membrane, and subsequently the inner mitochondrial membrane, of two mitochondria. GTPases of the dynamin superfamily — Mitofusin-1 (MFN1), MFN2, and OPA1 — are key players in fusion. MFN1 and MFN2 are located on the outer mitochondrial membrane and necessary for outer membrane fusion, whereas OPA1 resides in the inner membrane and is important for inner membrane fusion. Fusion leads to elongation of mitochondria under physiological conditions, which can help to maintain oxidative phosphorylation[87]. These GTPases can play a role in mitochondrial energetics. For example, the deletion of MFN2 in mice causes a deficiency in coenzyme Q, an electron carrier in Complex III, which causes a deficiency in the ETC and a decrease in ATP production[88]. Activation of these mitofusins and the cleavage of Opa1 can be regulated by changes in metabolism (see below).

Mitochondrial outer membranes are tethered by MFN1 and MFN2 dimerization and external stimuli such as oxidative stress can enhance outer membrane fusion[89]. The activation of inner membrane fusion can be regulated by changes in metabolism at the sites of proteolytic cleavage of OPA1[90]. OPA1 usually exists in a soluble long form and it can be cleaved by the ATP-dependent zinc metalloprotease YME1L or by the metalloendopeptidase OMA1, which is activated in response to a loss in membrane potential, to yield a soluble short form[82]. The soluble long and short forms are necessary for fusion to occur. During steady state, both can coexist to impart minor structural remodeling of mitochondria[91,92] The activation of cleaved OPA1 requires the presence of

GTP and the availability of GTP to activate OPA1 correlates with ATP levels in the cell[93,94]. The exact mechanism by which outer membrane and inner membrane fusion events are coordinated is still under investigation.

Fission is necessary to isolate damaged mitochondria from the network. The resulting daughter mitochondria may be unbalanced and the depolarized daughter mitochondrion is targeted for mitophagy[95] to sustain a population of healthy mitochondria (see below). However, excessive fission, as seen in diseases such as DN and AKI, can have harmful effects on mitochondrial homeostasis long term[96]. *In vitro* studies have been conducted to elucidate the mechanisms that trigger mitochondrial fission. Cells exposed to an excess of nutrients or oxidative stress have fragmented mitochondria[96]. Fission is induced by the translocation of DRP1 from the cytosol to the mitochondrial outer membrane as a result of a loss in mitochondrial membrane potential. If the membrane potential is not restored, mitochondria will be degraded via mitophagy[96]. DRP1 oligomerizes on the outer membrane to form a ring like structure around the mitochondria that can cause scission of the membrane[97]. DRP1 can bind to several different receptors, such as mitochondrial fission 1 (FIS1), mitochondrial dynamics proteins MID49 and MID51, and mitochondrial fission factor (MFF) that reside on the outer membrane[78]. DRP1 accumulates on the outer mitochondrial membrane by binding to these receptors and mediates the scission of mitochondria, a GTP dependent process[98]. MID51 contains a cytosolic domain with affinity for ADP and GDP, and can therefore act as a metabolic sensor[99,100]. DRP1 activity can be regulated by post-translational modifications such as phosphorylation, ubiquitylation, and sumoylation[101], and several signalling pathways have been shown to

regulate the phosphorylation of DRP1[102]. For example, phosphorylation of DRP1 at Ser637 by PKA inhibits its GTPase activity and, thus, inactivates fission[78,103]. By contrast, dephosphorylation of DRP1 at Ser637 by calcium and calmodulin dependent serine/threonine-protein phosphatase 2B catalytic subunit alpha isoform or calcineurin (CaN) activates DRP1 and promotes fission[104,105]. The balance between fission and fusion to maintain a functional population of mitochondria is an intricate process and is still under investigation. However mitochondria, such as damaged mitochondria, that disrupt this balance between fission and fusion are removed from the network via mitophagy.

[H3] Mitophagy.

Mitophagy in most cell types is regulated by the PTEN-induced putative kinase 1 (PINK1)–PARKIN mechanism that tags mitochondria for degradation[106]. PINK1, a kinase that is located in the mitochondria, is imported into the mitochondria and then degraded under physiological conditions[107]. As protein import is dependent on the mitochondrial membrane potential, mitochondrial depolarization results in the accumulation of PINK1 on the outer membrane; the PINK1-mediated phosphorylation of certain proteins here mediates the recruitment of the E3 ligase PARKIN[108-111] to the outer membrane. PARKIN attaches ubiquitin to lysine residues in the N termini of mitochondrial outer membrane proteins, such as MFN1 and MFN2, which targets the mitochondria for degradation by autophagosomes[112-116].

Several pathways regulate mitophagy (FIG. 1.5). Proteins that are important for autophagy, such as ULK1 and ULK2, can mediate mitophagy under different stimuli[117]. For example, when nutrients are sufficient, AMPK is inhibited and mTOR inhibits ULK1, suppressing mitophagy[118]. During nutrient deprivation, AMPK is activated and inhibits mTOR, allowing for ULK1 activation and mitophagy[117] (FIG. 3). Under oxidative stress, AMPK can be activated and inhibit mTOR, again activating mitophagy[52,118]. A more direct role for AMPK in the activation of mitophagy has also been suggested[119]. The authors of this study proposed that AMPK directly phosphorylates MFF on Ser155 and Ser172, triggering fission and, subsequently, mitophagy[120]. However, external stimuli that trigger this pathway are unknown and more research is needed.

Other stimuli, such as hypoxia, cause the serine/threonine-protein phosphatase PGAM5 to dephosphorylate its substrate, the mitophagy receptor, FUN14 domain-containing protein 1 (FUNDC1)[121]. FUNDC1 then interacts with microtubule-associated protein 1 light chain 3 (LC3), which mediates the formation of an autophagic membrane[121,122]. Alternatively, hypoxia can induce mitophagy through the actions of BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3) and BCL2/adenovirus E1B 19 kDa protein-interacting protein 3-like - (NIX) via a mechanism that involves HIF-1α[123,124]. HIF-1α can directly induce the transcription of BNIP3 and NIX by binding to the promoter of the gene encoding BNIP3 and by recruiting other coactivator proteins to the gene encoding NIX. NIX and BNIP3 are transmembrane proteins located in the mitochondrial outer membrane and they can activate mitophagy by dissipating the

mitochondrial membrane potential and interacting with LC3 to deliver mitochondria to the autophagosome[124-127]. BNIP3 and NIX are also apoptotic regulators that can induce cell death or autophagy by increasing the production of ROS, binding to pro-apoptotic proteins of the BCL2 family, or by inhibiting the GTP-binding protein RHEB, an upstream activator of mTOR[128-130]. Previous studies suggest that crosstalk exists between both of the mechanisms that can regulate mitophagy[124,131,132], although the mechanisms of this proposed crosstalk are unclear and additional studies are needed to determine the mechanisms that regulate mitophagy in renal diseases.

[H1] Mitochondria and renal diseases

Diseases such as Acute Kidney Injury (AKI) and Diabetic Nephropathy (DN) can cause an imbalance in mitochondrial homeostasis, negatively impacting mitochondrial energetics or the production of ATP. Much research has recently supported the role for mitochondrial dysfunction in a number of renal diseases such as CKD, diabetes, and glomerular diseases[133]. Although this dissertation will focus on AKI, we include both AKI and DN as examples of how mitochondrial dysfunction can negatively impact mitochondrial energetics to contribute to disease progression.

[H2] Acute kidney injury

The outcome of AKI is renal dysfunction, as indicated by an increase in blood urea nitrogen (BUN) and serum creatinine, and/or reduced urinary output [134]. However in the last two decades the criteria for identifying renal dysfunction caused by AKI has been continuously reassessed beginning with establishment of the risk, injury, failure, loss, and end-stage renal disease (RIFLE) criteria in 2004[135]. The AKI network (AKIN) included the absolute change in serum creatinine and modified the RIFLE criteria[136]. In recent years, both sets of criteria were combined to establish the 2012 Kidney Disease: Improving Global Outcomes (KDIGO) as shown in Figure 1.6[137]. This criterion describes three stages of AKI based on serum creatinine and urinary output. Although this criteria may prove to be more useful compared to those used in the past, ultimately, the treatment of AKI depends on its pathogenesis, cause of onset, and efficient early detection methods.

The pathogenesis is complicated and the onset is multifactorial[138,139]. Over the years, the incidence of AKI has increased and the mortality rate for patients that require renal replacement therapy is over 60%[134,140-143]. Ultimately, unresolved AKI can cause long term damage to the kidney, increasing the risk of chronic kidney disease (CKD)[144]. The etiology of AKI can be categorized as prerenal, postrenal, and intrinsic AKI[139]. Prerenal AKI is reversible and a cause of dysfunctional blood flow and glomerular hydrostatic pressure resulting in decreased filtration capacity[145]. Post renal AKI is a direct result of an obstruction of urinary flow and requires an ultrasound or equivalent methods to identify the obstruction of one or both ureters[145,146]. The pathophysiology of intrinsic AKI can result from sepsis, ischaemia–reperfusion injury (IRI), exposure to nephrotoxic reagents and trauma[147]. AKI can also result as a secondary insult in response to decreased

cardiovascular function[148,149]. One of the main sites of injury in AKI is the proximal tubules, which is characterized by disrupted brush borders and tight junctions, cell sloughing, apoptosis, necrosis, and the subsequent backleak of filtrate across injured proximal tubular cells[150].

Current detection methods, although are useful, are not efficient or accurate for detecting AKI in its early stages, prior to when KDIGO standards are applied. Current methods include testing for Kidney Injury Marker-1, KIM-1, and neutrophil gelatinase-associated lipocalin (NGAL). Both proteins are released from proximal tubules whereas NGAL can also be released from the distal tubule and both can be measured in the urine. Expression for NGAL can be detected 3 hours after injury whereas KIM-1 can be measured up until 24 hours after insult[145]. The release of KIM-1 and NGAL suggests that both proteins play a role in activating the immune system after injury[145]. KIM-1 triggers pathways necessary for the clearance of dead cells and remodeling of injured cells whereas NGAL can activate protective enzymes that prevent further damage to already injured cells[151,152].

Class	GFR	UO
Risk	↑ SCr × 1.5 or ↓ GFR >25%	<0.5 mL/kg/h × 6 h
Injury	↑ SCr × 2 or ↓ GFR >50%	<0.5 mL/kg/h × 12 h
Failure	↑ SCr × 3 or ↓ GFR >75% or if baseline SCr ≥353.6 μmol/L(≥4 mg/dL) ↑ SCr >44.2 μmol/L(>0.5 mg/dL)	<0.3 mL/kg/h × 24 h or anuria × 12 h
Loss of kidney function	Complete loss of kidney function >4 weeks	
End-stage kidney disease	Complete loss of kidney function >3 months	

[a]GFR, glomerular filtration rate; UO, urine output; SCr, serum creatinine.

Figure 1.6: Risk, Injury, Failure, Loss of kidney function and End-stage kidney disease (RIFLE) classification. From: Hertzberg, D., Rydén, L., Pickering, J. W., Sartipy, U. & Holzmann, M. J. Acute kidney injury-an overview of diagnostic methods and clinical management. Clinical kidney journal 10, 323-331, doi:10.1093/ckj/sfx003 (2017).

Much research has been conducted on mitochondrial dysfunction as an initiator and contributor of renal injury and as a therapeutic target[153,154] (Figure 1.7). Histologically, mitochondrial swelling and fragmentation are observed after diverse insults to the kidney[154]. A decrease in ATP production, an increase in ROS production, the release of cytochrome c, and the disruption of mitochondrial cristae are also observed, supporting a role for mitochondria in AKI [154]. A decrease in ATP production and mitochondrial dysfunction has been documented in many animal models of AKI, including sepsis, and these outcomes result from the loss of mitochondrial respiratory proteins in proximal tubules [155-157]. Furthermore, the loss of ETC proteins is persistent in the damaged kidney and this may contribute to the slow recovery of renal function after AKI[155].

In the ischemic kidney, a number of factors disrupt the oxidation and transport of fatty acids, causing an accumulation of fatty acids in the cytoplasm and, subsequently, contributing to the decrease in ATP production and mitochondrial energetics[158,17,154,159,160]. For example, cofactors such as NAD^+ are necessary for fatty acid oxidation but a dysfunctional ETC is not able to regenerate NAD^+ [161]. IRI also decreases the activity of CPT1[17,162], the rate-limiting enzyme in the carnitine shuttle that transports fatty acids from the cytoplasm into the mitochondria[162]. This decreases the transport of fatty acids into the mitochondria and reduces β-oxidation[162].

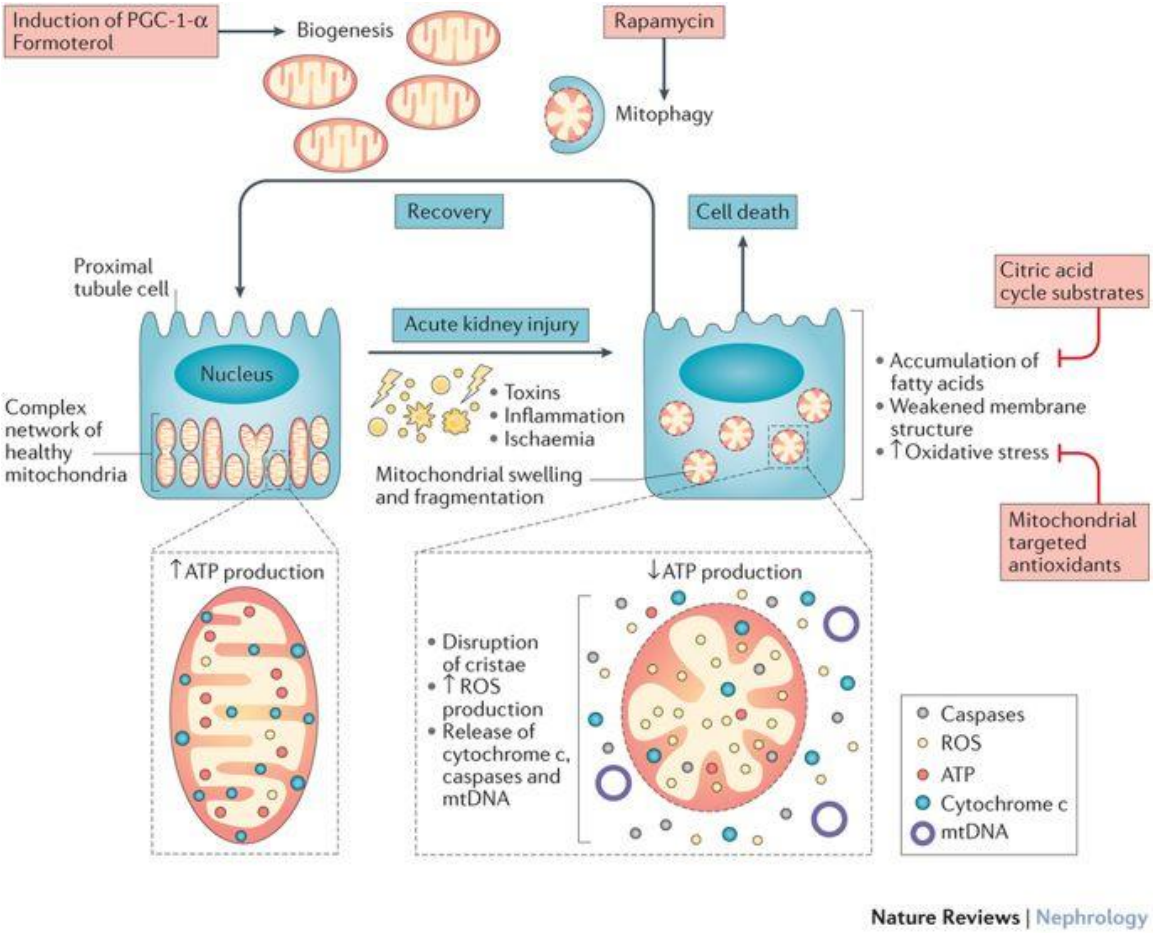

Figure 1.7: Mitochondrial injury and recovery during acute kidney injury (AKI)
From: Emma, F., Montini, G., Parikh, S. M. & Salviati, L. Mitochondrial dysfunction in inherited renal disease and acute kidney injury. *Nat Rev Nephrol* **12**, 267-280, doi:10.1038/nrneph.2015.214 (2016).

After IRI increased levels of lactate and pyruvate and of hexokinase activity in the kidney were reported, suggesting that there is an increase in glycolysis after injury[163,164]. Increased levels of glycolytic enzymes were also detected in injured renal tubules after IRI[165,166]. Thus, the kidney can respond to injury by altering its metabolic substrates to maintain function[167]. Further studies are needed to explore how this increase in glycolysis affects mitochondrial function in the kidney and if this change in metabolism contributes to recovery following IRI long term.

Another major contributor to the decrease in mitochondrial energetics seen in AKI are changes in mitochondrial dynamics[168] (FIG. 1.8). The translocation of DRP1 into the mitochondrial outer membrane occurs shortly after kidney injury[155,169]. Activation of DRP1 in ischemic kidneys promotes mitochondrial fragmentation and apoptosis[170]. Loss of cristae structure is also observed in AKI, dissipating the mitochondrial membrane potential and halting ATP production[154]. Administration of a pharmacological inhibitor of DRP1, mdivi-1, protected kidneys from AKI by inhibiting mitochondrial fragmentation, supporting a role for altered mitochondrial dynamics in AKI[169].

Mitophagy is also activated after ischemic AKI. In mice where autophagy regulator autophagy-related (ATG) 7 and ATG5 were specifically knocked out in renal proximal tubules, mitochondrial dysfunction was greater in renal proximal tubules in response to IRI, as characterized by severe morphological changes, increased ROS production and apoptosis[171-173]. The activation of NIX and BNIP3 causes the release of ROS and of the pro-apoptotic proteins BAX and BAK, in hypoxic conditions[113,174]. Deletion of BAX and

BAK in mouse kidneys not only protected mice from ischemic AKI but also suppressed the release of Cytochrome c and mitochondrial fragmentation, preserving mitochondrial integrity[175]. The lack of ATG7, also exacerbated cisplatin-induced AKI in mice[131,171]. These studies suggest that there is crosstalk between the mechanisms of cell death and autophagy in the activation of mitophagy.

The role of SIRT3 in cisplatin-induced AKI has also been explored. SIRT3 is a mitochondrial specific protein deacetylase with an active role in mitochondrial function and integrity[176]. An *in vitro* study using cisplatin-injured human renal proximal tubules showed that the overexpression of SIRT3 decreased the translocation of DRP1 from the cytosol to the mitochondrial outer membrane and thus, mitochondrial fission, providing a role for SIRT3 in regulating mitochondrial dynamics after AKI[176]. The deletion of SIRT3 exacerbates injury in a cisplatin-induced AKI mouse model, supporting its role in the recovery from this disease[176].

In mouse models of AKI, the transcription and protein expression of PGC-1α are suppressed persistently but are eventually restored to basal levels in models of AKI recovery[155]. As PGC-1α can regulate the transcription of mitochondrial proteins, the level of these proteins is also decreased after AKI[155,177].. In a model of septic AKI, global PGC-1α knockout mice showed a greater increase in BUN and creatinine levels than wild-type mice[156]. Renal specific PGC-1α knockout mice exhibited persistent AKI[156]. In contrast, the overexpression of PGC-1α in renal proximal tubule cells attenuates oxidant

injury *in vitro* [56]. Together, these studies show that PGC-1α is necessary for the recovery of renal function in AKI.

Investigations into the mechanisms by which PGC-1α regulates the recovery from AKI revealed a role for PGC-1α in NAD biosynthesis. Levels of nicotinamide, a precursor for NAD, were decreased after AKI in PGC-1α knockout mice and supplementation with nicotinamide reversed ischemic AKI[178]. We have reported that drugs/chemicals can upregulate MB by increasing PGC-1α protein expression in the recovery phase following IRI through two classes of G-protein coupled receptors (GPCRs): the β_2 adrenergic receptors and the 5-hydroxytryptamine 1F receptor[179,180] (see below).

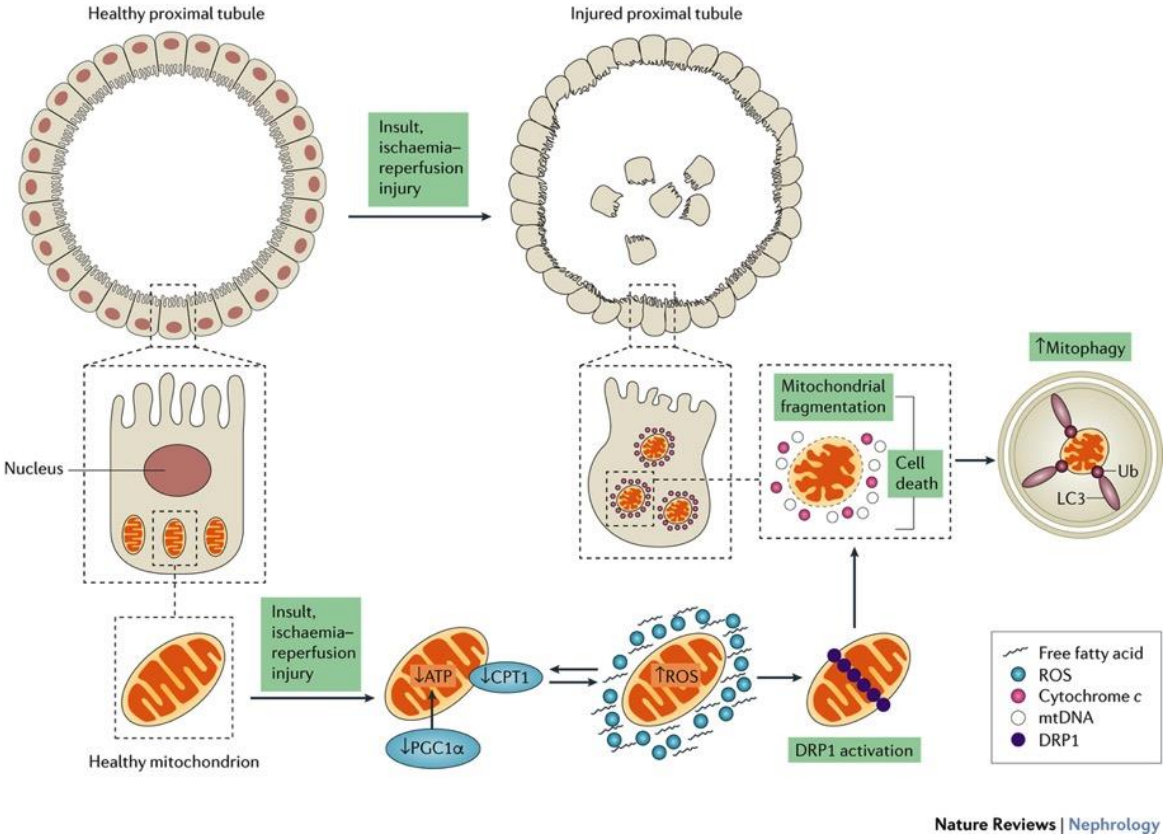

Figure 1.8: Changes in mitochondrial morphology lead to tubular damage in acute kidney injury
A healthy proximal tubule consists of an intact brush border with tight junctions and contains a network of mitochondria to maintain its function. After ischaemia–reperfusion injury (IRI), changes in mitochondrial function and morphology lead to mitochondrial dysfunction, and eventually to injured proximal tubules. In the early stages of acute kidney injury (AKI), a number of events may happen concurrently to cause a decrease in ATP production. These events include a decrease in the expression of carnitine O-palmitoyltransferase 1 (CPT1) (causing fatty acid accumulation and decreasing β-oxidation for ATP production), a decrease in the expression of peroxisome proliferator-activated receptor-γ co-activator 1α (PGC1α) and an increase in the production of reactive oxygen species (ROS) (bidirectional arrows). Together, these events can trigger the activation and accumulation of dynamin 1-like protein (DRP1) on the mitochondrial outer membrane, promoting mitochondrial fragmentation and eventually cell death. The release of cytochrome c and mitochondrial DNA (mtDNA) from dysfunctional mitochondria causes an increase in mitophagy. Mitochondrial dysfunction can induce cell death in injured proximal tubules, resulting in the loss of nuclei and tight junctions and in disrupted brush borders. Apoptotic or necrotic tubules can lead to cell sloughing, as seen in the centre of the tubule. From: Bhargava, P. & Schnellmann, R. G. (2017) Mitochondrial energetics in the kidney *Nat. Rev. Nephrol.* doi:10.1038/nrneph.2017.107

[H2] Diabetic nephropathy

DN is the leading cause of end-stage renal disease (ESRD) in the United States[181,182]. It is characterized by an increase in hyperglycemia, albuminuria, the accumulation of extracellular matrix proteins and glomerular and tubular epithelial hypertrophy, as well as a reduced glomerular filtration rate (GFR)[183]. Mitochondrial energetics are altered in DN due to a combination of increased ROS and hyperglycemia[184], both of which induce changes in the ETC that cause a decrease in ATP production and an increase in apoptosis[184]. In line with these observations, increased fission, mitochondrial fragmentation, and reduced levels of PGC-1α are all observed in the early stages of diabetes[185,186]. Structural changes in mitochondria correlate with the changes seen in energetics[186].

Hyperglycemia is the main contributing factor of DN (FIG. 1.9). Hyperglycemia increases the production of NADH and $FADH_2$ by the TCA cycle, fueling the ETC. ROS released from the ETC can damage mtDNA, hindering the production of mitochondrial proteins[187]. Originally it was thought that the excess glucose in hyperglycemia creates hyperpolarized mitochondria that produce more ATP and release superoxide from complexes I and III, causing mitochondrial dysfunction[184,188,189]. However, administering antioxidants such as vitamin E and vitamin A did not attenuate the complications seen in patients with diabetes, suggesting that mitochondrial ROS may not be the primary mediator of mitochondrial dysfunction in DN[190]. Hyperglycemia can also increase the level of advanced glycation end (AGE) precursors, the activity of the protein kinase C

(PKC) and hexosamine pathways, which can contribute to mitochondrial dysfunction[191]. All three events cause deleterious effects that include increased fibrosis, thrombosis, oxidative damage, and abnormalities in vasculature and blood flow[191].

Hyperglycemia also causes the conversion of glucose to fructose via the polyol pathway in proximal tubules, leading to ATP depletion[192]. A role for endogenous fructose metabolism in the regulation of DN was suggested by a study showing that knocking out the gene encoding fructokinase (*KHK*$^{-/-}$ mice), the enzyme responsible for the conversion of fructose to fructose-1-phosphate, in mice, protected them from DN induced by streptozotocin[193]. Proximal tubules are a major site of fructokinase expression[192,194] and ATP levels were increased and tubular morphology was improved in *KHK*$^{-/-}$ diabetic mice compared to wild type mice, suggesting a role for fructose metabolism in the pathogenesis of DN[193].

Mitochondrial fragmentation has been observed in proximal tubules in the early stages of diabetes [185], although the mechanisms that drive changes in mitochondrial dynamics in diabetes are still not clear. Fission dissipates the mitochondrial membrane potential, decreasing the production of ATP and promoting apoptosis[195]. Several studies have suggested a role for Rho-associated protein kinase 1 (ROCK1) signalling in activating fission in the diabetic kidney[196]. ROCK1 promotes the translocation of DRP1 to the mitochondria and triggers fission by phosphorylating DRP1[196]. Deletion of ROCK1 in streptozotocin-induced diabetes prevents fission, attenuates the increase in ROS production and restores bioenergetic function in the kidney[196].

In patients with diabetes, reduced levels of the fusion protein MFN2 were discovered[197]. In line with this finding, in kidneys from rats with streptozotocin-induced diabetes, the overexpression of MFN2 has a protective role in DN[197]. MFN2 overexpression decreased ROS production, decreased kidney volume and attenuated the pathological changes seen in the diabetic kidney[197]. Another protein that plays a role in fusion is called induced-in-high glucose 1 (IHG-1). Studies have reported that IHG-1 is a regulator of mitochondrial dynamics and biogenesis in the diabetic kidney[198]. IHG-1 can enhance the ability of MFN2 to bind to GTP and it directly interacts with MFN2 to mediate fusion[198]. Inhibition of IHG-1 reduces ATP production and hinders fusion *in vitro*[198]. IHG-1 has also been found to stabilize PGC-1α activation[199].

Reduced levels of PGC-1α were also observed in diabetic rat kidneys[200]. The overexpression of PGC-1α in mesangial cells *in vitro* attenuated the pathophysiological changes induced by hyperglycemic conditions[200]. The decrease in MB in diabetic rat kidneys is consistent with the translocation of DRP1 to the mitochondrial outer membrane and an increase in mitochondrial fragmentation[200]. It was also shown that the levels of PGC-1α mRNA and protein were reduced in podocytes cultured in hyperglycemic conditions compared to in podocytes cultured under normal glucose conditions, indicating a decrease in MB[201].

Another study recently reported the importance of pyruvate kinase M2 in DN. In diabetic patients that have not developed DN, pyruvate kinase M2 (Pkm2) expression and activity is upregulated but not in DN patients[202]. Investigators observed a decrease in PGC-1α mRNA and mitochondrial mass in *Pkm2*-knockdown podocytes[202]. Activation of Pkm2 attenuated the decrease in mitochondrial function and glycolytic flux in podocytes *in vitro*. *In vivo* studies showed that the activation of Pkm2 in mice attenuated the decrease in PGC-1α mRNA and increased the expression of OPA1, increasing fusion[202]. As such, activation of Pkm2 can reverse mitochondrial dysfunction and therefore renal abnormalities delaying the progression to DN. These studies show the need for more research, as targeting the balance between MB and dynamics could be a potential therapeutic target for DN.

Mitochondrial dysfunction is a major contributor to the progression of renal disease, in particular AKI and DN, as described above and shows promise as a potential target for the treatment of renal disease.

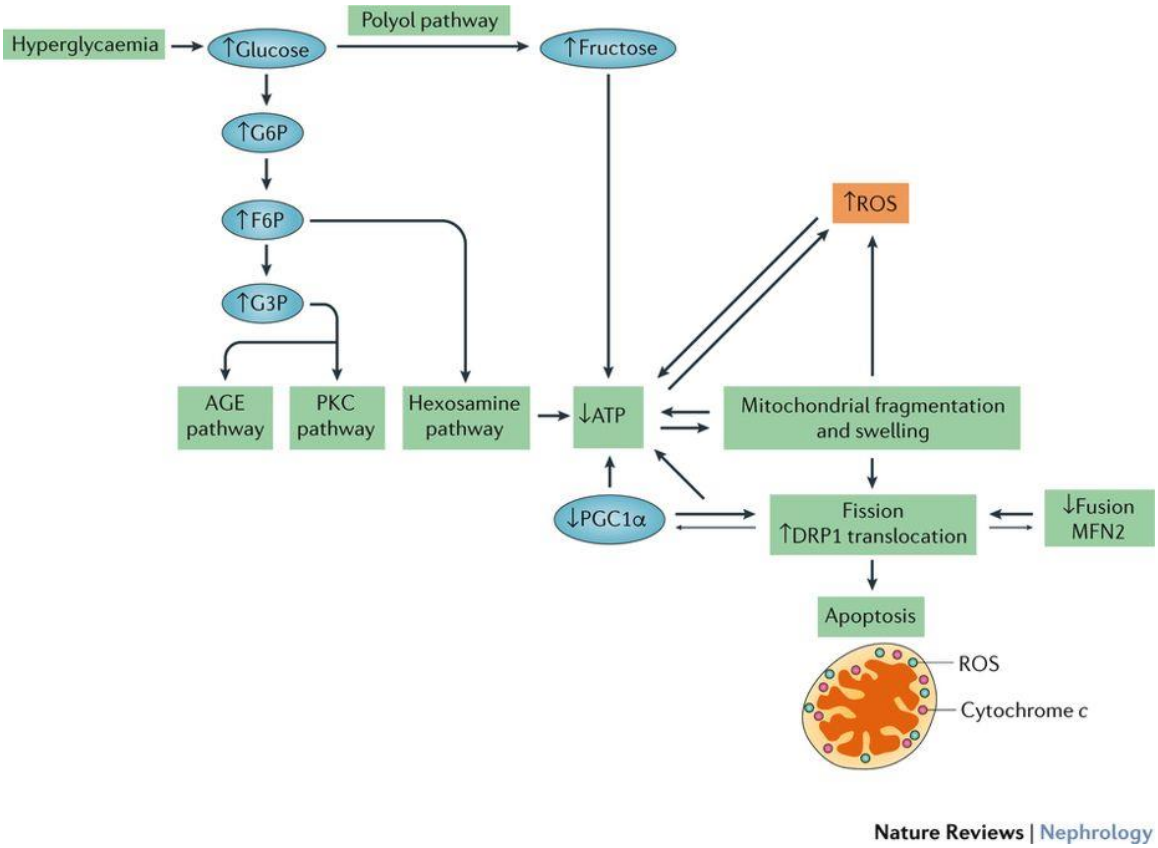

Fig 1.9: Factors contributing to mitochondrial dysfunction in diabetic nephropathy Hyperglycaemia is the primary contributing factor to mitochondrial dysfunction in diabetic nephropathy. An increase in glucose level results in an increase in glycolysis, in turn activating the advanced glycation end product (AGE) pathway, the protein kinase C (PKC) pathway and the hexosamine pathway, which results in a decrease in ATP levels. Hyperglycaemia also activates the polyol pathway, which increases fructose levels and, consequently, decreases ATP levels. Mitochondrial fragmentation and swelling is observed in early diabetic nephropathy, leading to an increase in fission and the production of reactive oxygen species (ROS). The correlations between increased mitochondrial fragmentation and decreased ATP, and between ROS production and decreased ATP, are interdependent. Whether one causes the other is unclear, as depicted by the bidirectional arrows. Decreases in the levels of mitofusin 2 (MFN2) and peroxisome proliferator-activated receptor-γ co-activator 1α (PGC1α) correlate with, and might contribute to, the increase in mitochondrial fission observed in diabetic nephropathy, as indicated by the larger arrows pointing towards increased mitochondrial fission. Decreases in mitochondrial energetics that are caused by changes in mitochondrial morphology and hyperglycaemia lead to apoptosis in diabetic nephropathy. F6P, fructose-6-phosphate; G6P, glucose-6-phosphate; G3P, glyceraldehyde-3-phosphate. From: Bhargava, P. & Schnellmann, R. G. (2017) Mitochondrial energetics in the kidney *Nat. Rev. Nephrol.* doi:10.1038/nrneph.2017.107

[H1] Mitochondrial energetics and therapy

[H2] Targeting AMPK signaling

AMPK signalling has been implicated as a target for correcting metabolism and mitochondrial function, especially in the kidney. As mentioned before, AMPK is a metabolic sensor for ATP in the cell. High AMP/ATP activate AMPK to stimulate cell growth and cellular metabolism. The AMPK activator 5-aminoimidazole-4-carboxamide-1-β-d-riboside (AICAR), prevents glomerulopathy and tubulointerstitial fibrosis in mice by stimulating fatty acid oxidation (FIG 1.10)[203]. AICAR also has a therapeutic effect in mouse renal IRI and can improve glucose utilization in obese, insulin-resistant rats[204,205]. The activation of AMPK by AICAR increased the level of PGC-1α levels and of mitochondrial proteins while reducing ROS production in a diabetic mouse model[206].

Several studies have suggested that there is crosstalk between AMPK activation and SIRT3 signalling [207,208]. SIRT1 and SIRT3 are activated by NAD^+. In cisplatin treated mice, SIRT3 gene and protein expression were decreased, tubular damage was increased, and the level of phosphorylated AMPK was decreased compared to control mice treated with saline.[209]. The administration of AICAR, to cisplatin treated mice attenuated the decrease in SIRT3 protein and gene expression, the level of phosphorylated AMPK, and tubular damage[209]. These studies provide a therapeutic role for targeting AMPK signalling in the kidney for improved outcomes in AKI and DN.

[H2] Targeting PPARs

As PPARs can regulate cellular metabolism, mitochondrial function, MB, fatty acid oxidation and glucose homeostasis, targeting them for treatment for renal diseases could be beneficial.

The activation of PPARs can play a role in ameliorating ischemic AKI[210-212]. As discussed previously, an accumulation of fatty acids and increased ROS production can decrease the efficiency of the ETC. Defects in fatty acid oxidation were attributed to the down regulation of PPARs during renal ischemia[17]. Fenofibrate, a drug used for treating dyslipidemia, activates PPARα (Figure 1.10)[213]. The downstream effect of targeting PPARα is the activation of lipoprotein lipase, which hydrolyzes triglycerides into glycerol and free fatty acids for metabolism[213]. PPARs can also stimulate MB; for example, compounds such as bardoxolone increase the level of PPARγ and NRF2 mRNA, leading to MB[214]. However, the use of bardoxolone in clinical trials for type 2 diabetes and stage 4 CKD showed adverse effects in patients including an increase in heart failure events and led to the termination of the trial[215].

PPARs agonists also show promise as treatment for DN based on their efficacy in animal models. Previously, studies treating *db/db* diabetic mice with fenofibrate showed that it decreased hyperglycemia and insulin resistance, potentially by correcting glucose homeostasis.[216] Studies have also shown a decrease in fatty acids in the kidney upon treatment of a diabetic mouse model with fenofibrate, supporting its potential as a

therapeutic for DN[217-219]. These *in vivo* studies provide evidence that fenofibrate might be suitable to treat patients with DN. Indeed, fenofibrate decreased dyslipidemia and albuminuria in patients with type II diabetes and reduced further cardiovascular events[220]. Taken together, these studies confirm that PPARs have a role in DN and are a therapeutic target.

[H2] Targeting G protein-coupled receptors

Although a wide variety of GPCRs are expressed in the kidney, few studies correlate GPCRs with mitochondrial function in the kidney and other organs. We proposed that compounds that target two different classes of GPCRs — β-2 adrenergic receptors (β2AR) and 5-hydroxytryptamine receptor 1F (5-HT_{1F}) receptors— can induce MB, restore mitochondrial function and stimulate the recovery of renal function following IRI. Formoterol, a β2AR agonist used to treat asthma and chronic obstructive pulmonary disease, stimulates MB and the expression of PGC-1α in renal proximal tubular cells and in mice[221]. The administration of formoterol in a model of IRI AKI following renal injury accelerated the recovery of mitochondrial and renal function by six days[221]. LY344864 is a potent agonist for 5-HT_{1F} receptors and it induced MB in naïve mice and accelerated the recovery of MB and renal function in the same AKI model[180]. There are currently several agonists of GPCRs in clinical trials for the treatment of DN, such as atrasentan; however, whether they act by influencing mitochondrial energetics is unknown and requires further research. These studies provide a foundation for pursuing the targeting of

GPCRs, particularly β2AR and 5-HT_{1F}, as a treatment for mitochondrial dysfunction in renal diseases.

[H2] Using mitochondrial peptides

Recently a family of peptides called Szeto-Schiller peptides (SS peptides) were found to specifically target cytochrome c activity in the ETC, enhancing its efficiency and increasing 'state 3 respiration' — that is, ATP production in the presence of excess substrates and ADP[222]. SS peptides are highly polar, water soluble tetrapeptides that can cross the blood–brain barrier and specifically target the inner mitochondrial membrane. The SS peptides do not cause mitochondrial depolarization upon entry, making these compounds highly promising for treatment. SS peptides prevent the peroxidation of cardiolipin, a phospholipid important for maintaining cristae formation, by cytochrome c[222]. Cytochrome c binds and oxidizes cardiolipin, disrupting cristae formation and detaching cytochrome c from the inner mitochondrial membrane[223,224]. The SS-31 peptide has been shown, in a variety of animal disease models, especially in AKI, to promote ATP recovery and cristae formation[222]. Pre-treatment of rats with SS-31 *in vivo* maintained cristae formation and prevented mitochondrial swelling of renal tubular epithelial cells[222]. Due to the success in animal models, SS-31, which is also known as Bendavia, is currently in clinical trials for the treatment of AKI (Figure 1.10)[225].

Table 1 | **Approaches to correct abnormal mitochondrial function in AKI and diabetic nephropathy**

Agent	Mechanism of action	*In vivo* and clinical studies*
Acute kidney injury		
AICAR (an AMPK activator)	• Increases AMPK activation • Increases crosstalk between SIRT3 and AMPK	• AICAR administration attenuated decreased serum creatinine and urea levels in Sprague–Dawley rats with IRI (2012)[200] • AICAR attenuated BUN and serum creatinine levels in cisplatin-treated mice (2015)[206]
Formoterol (a β_2AR agonist)	Binds to β_2AR and induces mitochondrial biogenesis	Formoterol restored mitochondrial and renal function in mice with IRI within 6 days (2014)[174]
LY344864 (a 5-HT_{1F} receptor agonist)	Binds to 5-HT_{1F} and induces mitochondrial biogenesis	LY344864 restores renal function in mice with IRI within 6 days (2014)[175]
Elamipretide (a Szeto–Schiller peptide (specifically SS-31))	Prevents the peroxidation of cardiolipin by cytochrome *c*	• Enhances efficiency of the ETC and prevents mitochondrial swelling in rats (2014)[218] • Phase I study (NCT02436447) in patients with impaired renal function (2015)[221]
Diabetic nephropathy		
AICAR (an AMPK activator)	Increases glucose utilization	AICAR decreased blood glucose levels in *db/db* diabetic mice and *ob/ob* obese mice (2002)[228]
Fenofibrate (a PPARα agonist)	• Decreases hyperglycaemia • Increases free fatty acids by targeting lipase • Decreases dyslipidaemia and albuminuria	• Corrected glucose homeostasis in *db/db* diabetic mice (2006)[213] • Decreased serum creatinine levels and had a renoprotective role for diabetic nephropathy in diabetic rats (2016)[215] • Administrating fenofibrate to patients with type 2 diabetes mellitus decreased cardiovascular disease events (2005)[229]

5-HT_{1F}, 5-hydroxytryptamine receptor 1F; β_2AR, β_2 adrenergic receptor; AICAR, 5-aminoimidazole-4-carboxamide-1-β-D-riboside; AKI, acute kidney injury; AMPK, AMP-activated protein kinase; BUN, blood urea nitrogen; ETC, electron transport chain; IRI, ischaemia–reperfusion injury; PPARα, peroxisome proliferator-activated receptor-α; SIRT3, sirtuin 3. *The year of the clinical study is given in parentheses.

Figure 1.10: Approaches to correct abnormal mitochondrial function in AKI and diabetic nephropathy

From: Bhargava, P. & Schnellmann, R. G. (2017) Mitochondrial energetics in the kidney *Nat. Rev. Nephrol.* doi:10.1038/nrneph.2017.107

Soluble Guanylyl Cyclase

Nitric oxide, NO, is a small gaseous, nonpolar molecule that has been studied since the 1980's. Robert Furchgott discovered that the relaxation of blood vessels needs endothelial cells and that smooth muscles cells alone could not exhibit these effects[226]. Furchgott proposed that endothelial cells are secreting endothelial derived relaxing factor, EDRF, which is necessary for blood vessel relaxation[226]. Meanwhile, while elucidating the mechanism of action of nitroglycerin, Ferid Murad discovered that nitroglycerin causes the release of NO and that this causes the relaxation of smooth muscle cells[227]. Ignarro showed that EDRF and NO are the same molecule, piecing together the work of Murad and Furchgott[228]. Simultaneous work by these scientists resulted in a Nobel Peace Prize in physiology in 1998.

NO is produced by nitric oxide synthase (NOS). There are three types of NOS: endothelial (eNOS), neuronal (nNOS), and inducible (iNOS). NO is produced by oxidizing L-arginine to L-citrulline and requires Ca^{2+} as a cofactor, tetrahydrobiopterin (BH4), and NADPH to accomplish this task[229]. The main receptor for NO is Guanylyl Cyclase (GC). There are two forms of GC, soluble GC (sGC) and membrane bound particulate GC (pGC). sGC is found in the cytosol of most cell types and is the primary receptor for NO. The family of pGCs contain up to six identified isoforms termed as GC-A to GC-F[230]. Although both sGC and pGC have the same function in terms of converting GTP to cGMP, for the remainder of this dissertation, the focus will be on sGC.

sGC is a 150kD heterodimer consisting of the alpha and beta subunits. There are two types for each subunit. Depending on the cell type, different combinations of sGC heterodimers may exist as $\alpha_1\beta_1$, $\alpha_1\beta_2$, and $\alpha_2\beta_1$[231,232]. The $\alpha_1\beta_1$ heterodimer is found in most mammalian tissues including the heart and kidney. The sGC heterodimer consists of the heme-nitric oxide oxygen domain (H-NOX), PAS domain, coiled-coil domain, and cyclase domain, Figure 1.11[233]. The β_1-subunit is the catalytic subunit of sGC and contains the heme moiety bound to the residue, Histidine-105, located in the H-NOX domain at the N terminus of the β_1-subunit[234]. NO binds to the heme moiety in the β_1-subunit causing a conformational change in the enzyme resulting in a pentacoordinated Fe-NO complex[235]. This conformational change causes a break between Histidine-105 and the heme moiety activating the enzyme and causing the conversion of GTP to cGMP[236].

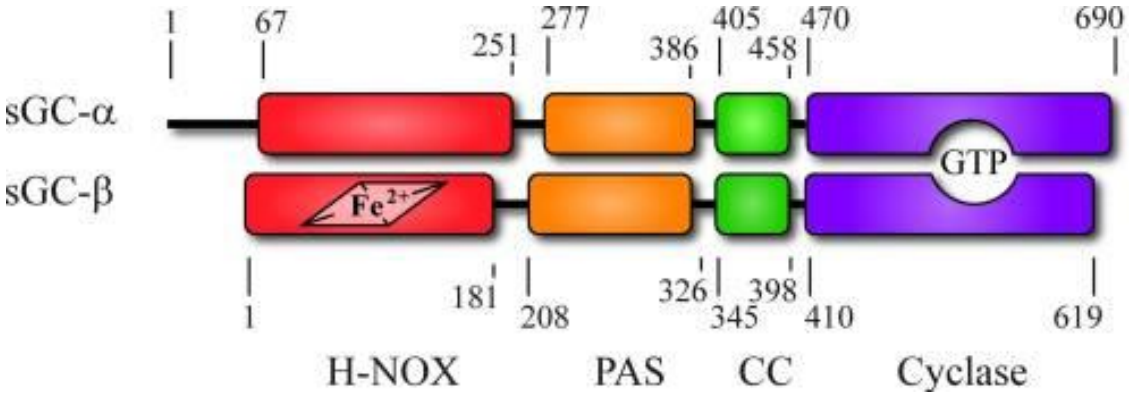

Figure 1.11: **sGC domain structure.** Shown are the approximate boundaries for each domain in sGC. Numbering is for the α1 and β1 subunits of human sGC. sGC, soluble guanylyl/guanylate cyclase. From : Montfort, W. R., Wales, J. A. & Weichsel, A. Structure and Activation of Soluble Guanylyl Cyclase, the Nitric Oxide Sensor. Antioxid. Redox Signal. 26, 107-121, doi:10.1089/ars.2016.6693 (2017)

NO-independent signaling

NO-dependent mechanisms operate primarily through sGC and pGC as described above and they involve cGMP to elicit most their effects. However, NO-independent mechanisms also exist and play a prominent role in disease. The function and activity of many proteins can be regulated by post-translational modification. The presence of NO can modify many proteins thereby regulating their function. NO can modify free thiols on cysteine residues in a process called S-nitrosation/nitrosylation[237,238]. As shown in the Figure 1.12, proteins can undergo S-nitrosation or S-nitrosylation as well as be denitrosylated, or removal of the NO group on the thiol[239]. Glutathione and thioredoxin systems are necessary for these processes. S-nitrosoglutathione (GSNO) reductase (GSNOR) denitrosylates proteins[240,241]. GSNOR may potentially play a role in regulating blood pressure. Inhibitors of GSNOR cause vasodilation and deletion of GSNOR causes an overall decrease in vascular resistance[242-244]. Although S-nitrosation occurs in normal physiological conditions, excessive oxidative or nitrosative stress can cause s-nitrosation of sGC. For example, treatment with nitroglycerin, in vivo, was shown to induce S-nitrosation of sGC[245]. This can decrease activity of sGC and desensitize sGC to surrounding NO. S-nitrosation of sGC can serve as a negative feedback loop and therefore decrease cGMP production[246]. On the other hand studies have shown that S-nitrosation may occur on cGMP phosphodiesterases, enzymes that degrade cGMP, and inhibit its activity therefore increasing cGMP levels[239].

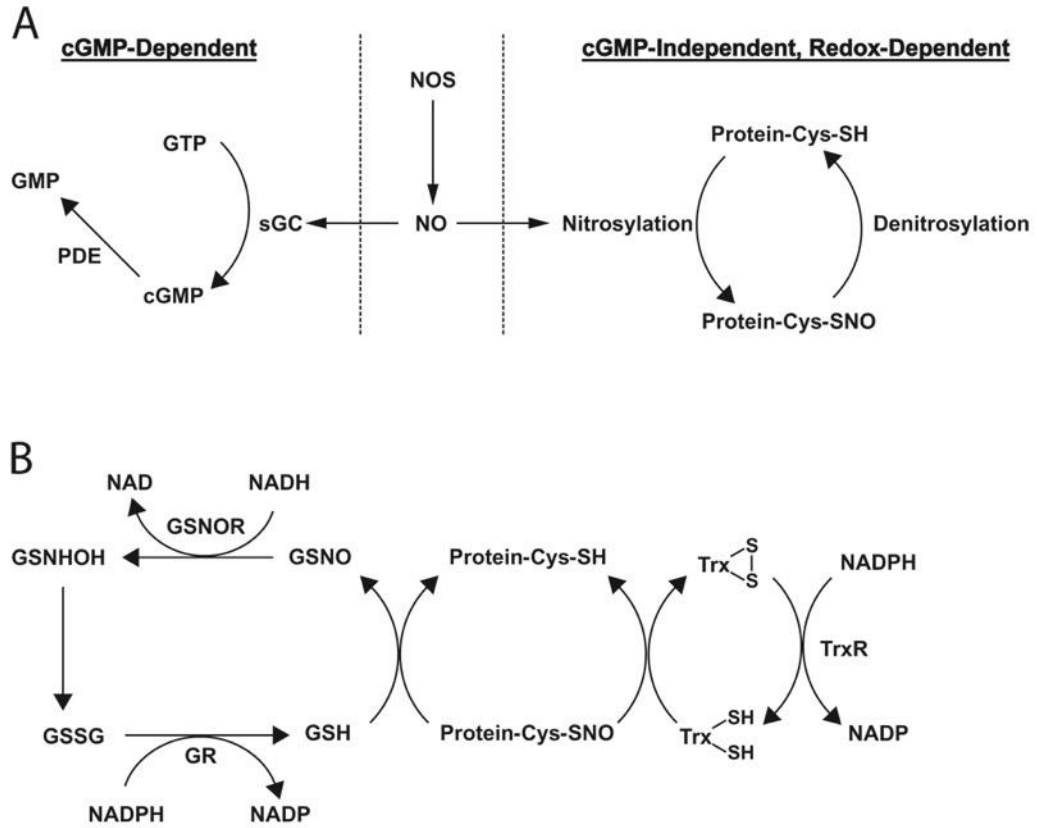

Figure 1.12: The roles of cGMP and S-nitrosylation in NO-based signaling (A) and enzymatic protein denitrosylation mediated by the S-nitrosoglutathione reductase (GSNOR) and thioredoxin (Trx) systems (B). (A) NO synthase (NOS) synthesizes NO, which may activate soluble guanylyl cyclase and thereby enhance production of cGMP (left) or subserve protein S-nitrosylation (right). The cGMP-dependent pathway is deactivated by cGMP-phosphodiesterase (PDE), which hydrolyzes cGMP to GMP (PDE may also be activated allosterically by cGMP). The SNO-based mechanisms are dynamically regulated via S-nitrosylation and denitrosylation of a multitude of cysteine-containing proteins. In contrast to the multiple elements regulated by S-nitrosylation, the cGMP-based signaling system relies primarily on the cGMP-dependent protein kinase, PKG. (B) Proteins undergo reversible S-nitrosylation and denitrosylation (center). Denitrosylation mediated by GSNOR is depicted on the left. Transnitrosylation of glutathione (GSH) by S-nitrosylated proteins generates GSNO and native protein. GSNO undergoes NADH-dependent reduction by GSNOR to generate glutathione S-hydroxysulfenamide (GSNHOH), which can undergo further reaction with GSH to generate oxidized glutathione (GSSG). The redox cycle is completed by reduction of GSSG to GSH via GSSG reductase. Denitrosylation mediated by the thioredoxin (Trx) system is depicted on the right. The active site dithiol motif (CXXC) of Trx1 (cytoplasmic) or Trx2 (mitochondrial) undergoes oxidation coupled to denitrosylation of SNO substrate. Oxidized Trx is reduced by the selenoprotein thioredoxin reductase (TrxR), which employs the reducing power of NADPH to regenerate active Trx. From: Lima, B., Forrester, M. T., Hess, D. T. & Stamler, J. S. S-nitrosylation in cardiovascular signaling. Circ. Res. 106, 633-646, doi:10.1161/circresaha.109.207381 (2010).

sGC and the Heart

Dysfunction in the eNOS/sGC/PKG pathway has been discovered and investigated as the cause in many diseases such as acute heart failure (HF), DN, ischemic diseases, 5/6 nephrectomy, anti-thy1 glomerulonephritis and others[247-249]. The amount of NO in the microenvironment of these diseases has pushed investigators to design and investigate compounds that can efficiently target sGC and maintain cGMP production. In the case of HF, there is an increase in oxidative stress that can affect the redox state of sGC[250]. The increase in ROS creates a toxic environment in the endothelium by scavenging for surrounding NO and forming peroxynitrite (ONOO-)[251]. The resulting ONOO- production occurs at faster rate than the rate for GPx to neutralize ONOO- levels, leaving the endothelium damaged[252]. ONOO- can damage DNA and modify proteins inhibiting their function, as well as deplete GSH levels needed for GPx function[252]. Originally, NO donors such as nitroglycerin were administered to combat the decrease in NO bioavailability and hopefully still stimulate cGMP production and therefore vasorelaxation of blood vessels[253]. However, administering nitroglycerin increased ONOO- levels and decreased downstream PKG signaling subsequently decreasing vasorelaxation[254].

To target cGMP production in environment with low NO bioavailability, investigators discovered the first sGC modulator, YC-1[255]. YC-1 could increase cGMP production efficiently and was used as a lead compound for the design of other modulators[256]. There are two classes of sGC modulators, sGC stimulators and activators. sGC stimulators such as Riociguat, target the NO-sensitive/reduced state of sGC sensitizing sGC to endogenous

levels of NO that may still be produced in the toxic environment. sGC activators such as Cinaciguat, target two forms of sGC: the NO-independent/oxidized state of sGC and hemeless sGC. The balance between reduced and oxidized sGC is complex and can shift towards oxidized sGC in these diseases. Excessive ROS in the endothelium can oxidize the heme moeity in sGC from its ferrous state to ferric and possibly remove the heme from the catalytic subunit completely[250,257,258]. The hemeless state of sGC can then be targeted for degradation[259]. Several investigators have studied the role of both riociguat and cinaciguat in cardiovascular diseases[260,261]. As a result, currently riociguat is in clinical trials for pulmonary arterial hypertension (PAH) as ADEMPAS and cinaciguat for HF[262,263]. Both drugs have also shown to play a role in renal diseases such as CKD and those results are summarized in several reviews[264,265].

cGMP Targets

cGMP phosphodiesterases

Figure 1.13, shows a detailed diagram with cGMP and its three classes of targets. Both cGMP and cAMP compete for the allosteric binding sites on phosphodiesterases (PDEs). There are a total of 12 types of PDEs where certain PDEs such as PDE5, PDE6, and PDE9 have a higher affinity for cGMP than cAMP[266]. The lack of cGMP production in certain diseased states has raised the question for the role in PDE inhibition[267]. Sildenafil is a PDE5 inhibitor that has shown to play a role in recovery in renal I/R and to elicit cardioprotective effects in cardiac I/R[73,268].

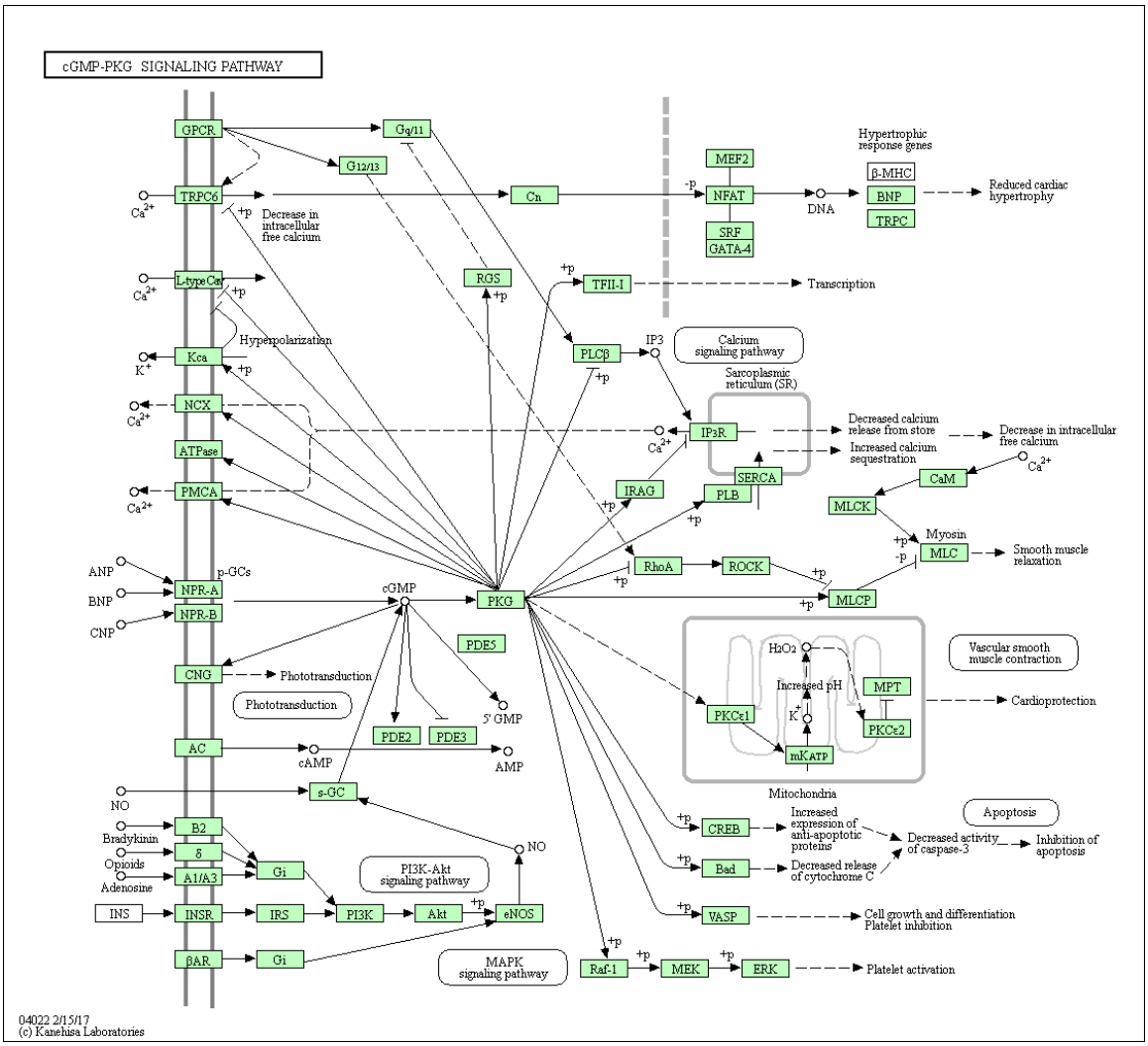

Figure 1.13: cGMP-PKG signaling pathway
From: Kyoto Encyclopedia of Genes and Genome (KEGG) 04022 02/15/17, copyright from Kanehisa Laboratories, https://www.genome.jp/kegg-bin/show_pathway?oas04022.

cGMP-Gated ion channels

The second class of targets is cyclic nucleotide gated ion channels (CNGs). CNGs can by targeted by cGMP and cAMP, however their distributions varies and are commonly associated with sensory signal transduction. There are at least three classes of CNGs: CNG-1, CNG-2, and CNG-3. Although CNGs are primarily associated with photoreceptors and olfactory neurons, they are expressed in a wide variety of organs such as the brain, heart, colon, pancreas, lung, and kidney[269]. CNG-1 and CNG-3 expression exists in the kidney[269]. CNGs found in rod and cone channels are activated by cGMP whereas olfactory channels are activated by both cGMP and cAMP[269]. CNGs expressed in other organs play a role in regulating permeability to calcium and monovalent ions such as Na^+ and K^+.

CNGs are non-selective ion channels that allow for the passage for both monovalent and divalent ions[270]. Their activity can be partly regulated by post-translational modifications such as phosphorylation and by the Ca^{2+} binding protein, calmodulin[270,271]. CNGs are not constitutively active and depend on the concentration of cGMP in the cell to stabilize a response[270]. CNGs in the kidney were found to contain characteristics in terms of activity, similar to CNGs in photoreceptors[272,273]. Upon further investigation, it was discovered that the cDNA for both types of CNGs share 99% nucleotide sequence identity and whatever minimal differences observed in cDNA, resulted in differences in function[273].

Specific examples of CNGs in the kidney include epithelial sodium channels (ENaC) and Na+K+ATPase pumps[274]. ENaCs are responsible for Na^+ reabsorption where upon binding cGMP can mediate effects of natriuresis, the excretion of sodium, and diuresis, the process of increasing urine volume[182,275,276]. Another CNG commonly expressed in the kidney as well as other organs is called Aquaporin-1 (AQP1), an osmotic water channel[277]. cGMP directly binds to the C-terminus of AQP1 and subsequently activating it and increasing water uptake[277].

Protein Kinase G

The third class of cGMP targets is called protein kinase G (PKG), a serine/threonine kinase. There are two types of PKG: PKGI and PKGII. PKGI can be further characterized into two isoforms, PKG1a and PKG1b. Both isoforms are produced as a result of alternative splicing of the PKG1 (prkg1) gene and differ in the N-terminal amino acids[278-281]. Both isoforms exist in a variety of tissues however the proportion of expression of both, is cell type dependent[281-283]. PKG1a has a higher affinity for cGMP than PKG1b by 10 fold[282,284,285]. Many studies have suggested both isoforms sharing substrates however, this depends on the basal level of cGMP produced in the cell. If high amounts of cGMP are available in the cell and PKG1a is activated, PKG1b phosphotransferase activity can be activated as well. PKG1a will be the focus of this dissertation regarding PKG[266].

PKG1 consists of a catalytic domain and a regulatory domain. The catalytic domain of PKG contains a subdomain that binds to Mg^{2+} and ATP as well as another subdomain that binds to substrates. cGMP binds to two allosteric sites in the regulatory domain and increases catalytic activity of PKG from 3 to 10-fold[280,281,284,286,287]. The regulatory domain contains an autoinhibitory domain and an extended leucine zipper region (ZZZZZ). In the autoinhibitory subdomain, catalytic residues directly bind to residues in the autoinhibitory domain, suppressing catalytic activity in the absence of cGMP[279,288]. The autoinhibitory domain contains an amino acid sequence that is phosphorylated by the catalytic domain, although this residue cannot be phosphorylated[266]. This sequence, ^{59}TRQAIS^{63}, is called a pseudosubstrate sequence, and the pseudo phosphorylation occurs on alanine[266]. Also in the catalytic domain, the leucine zipper region allows for dimerization of the PKG monomers and allows for specific targeting of certain proteins called cGMP-dependent protein kinase-interacting proteins (GKIPs)[266]. These interactions between PKG1 and GKIPs can help localize PKG to different parts of the cell and bind to its substrates[266]. Several examples of possible substrates of PKG1 include ATP-sensitive K+ channel[289], MEKK1[290], RhoA[291-293], and Vasodilator-stimulated phosphoprotein (VASP)[294,295]. An updated list of potential substrates can be found in in-depth reviews[266,280].

Although there are many substrates that are directly phosphorylated by PKG, there are several important proteins that can be considered as downstream targets that are associated with PKG. Much of the literature has shown a correlation between PKG and these associated downstream targets. These downstream targets include: AKT, ERK1/2,

Glycogen Synthase Kinase 3 β (GSK3β), and p38[266,268,296-299]. The following chapters will discuss the effect of ERK1/2 and p38 in detail.

[H1] Conclusions

Mitochondrial homeostasis involves a network of cellular processes that regulate ATP production and the disruption of any of these processes can result in mitochondrial dysfunction and organ damage. Although much is known about mitophagy, fission, fusion, and MB, the precise role of these processes in renal disease remains to be determined. However, it is clear that mitochondrial dysfunction is common and occurs early in AKI and DN. Furthermore, the absence of recovery of mitochondrial function after diverse insults may lead to the continued impairment of renal function that leads to chronic renal disease. Because renal cell repair and the recovery of renal function is dependent on ability of mitochondria to produce ATP, restoring mitochondrial function might reverse cellular injury and restore renal function. Collectively, these studies corroborate the need to target mitochondrial homeostasis to reverse the state of disease, restore mitochondrial function and stimulate organ repair or prevent further decreases in organ function.

Nisoli and group first suggested the role of NO in inducing mitochondrial biogenesis (MB)[71]. Moreover, this induction was also cGMP-dependent. Since this finding, there have been numerous studies in many different cell types regarding the cGMP-dependent pathway of inducing MB. In the kidney specifically, although many correlations have been made between cGMP and MB, the pathway is still undefined. Here, we will elucidate how sGC/cGMP signaling regulates MB in the renal proximal tubule cells and in renal I/R.

Chapter Two: Characterization of Soluble Guanylyl Cyclase Signaling in the suppression of Mitochondrial Biogenesis in Ischemia/Reperfusion-Induced Acute Kidney Injury

Introduction

Soluble Guanylyl Cyclase (sGC) is the most common receptor for nitric oxide (NO) converting GTP to cyclic GMP (cGMP). sGC is a 150 kD heterodimer consisting of the alpha 1 and beta 1 subunits where the beta 1 subunit is the catalytic subunit and contains the heme moiety bound to Histidine 105[234]. The heme moiety predominantly exists in its reduced or ferrous form, Fe^{2+}. Historically, sGC signaling begins with the generation of nitric oxide (NO) by one of three nitric oxide synthases (NOS): inducible, endothelial, and neuronal. The generation of NO by NOS occurs by oxidizing L-arginine to L-citrulline[300]. NO binds to the heme moiety, inducing a conformational change in the enzyme and creating a pentacoordinated Fe-NO complex[235]. This conformational change induces a break between Histidine 105 and the bound heme moiety, subsequently activating the enzyme for the conversion of GTP to cGMP[236]. Although picomolar amounts of NO can activate sGC, in the presence of excessive NO sGC activity can increase by 100-fold[301]. The resulting production of cGMP can activate cGMP-gated ion channels, phosphodiesterases (PDEs), and protein kinase G (PKG). Commonly discussed effects of sGC signaling include vasodilation, vascular smooth muscle relaxation, and platelet aggregation, and potentially regulating mitochondrial biogenesis (MB)[71].

Conditions such as oxidative stress can negatively affect sGC signaling by introducing reactive oxygen species (ROS) that can scavenge for NO causing decreased downstream signaling. Conditions of excessive ROS can cause the oxidation of the ferrous heme in sGC, to ferric (Fe^{3+}), allowing for inefficient production of cGMP. If conditions of excessive ROS persist, sGC can lose the heme moiety from its catalytic site creating the

heme-free state and rendering sGC completely inactive and vulnerable to degradation[281]. ROS can also target eNOS by inhibiting their function and decreasing overall NO bioavailability[302,303]. As a result, two classes of drugs were designed to pharmacologically target the reduced form of sGC or heme-dependent state and the oxidized form or heme-independent state. sGC stimulators, such as riociguat, target the reduced state of sGC by further sensitizing sGC and synergistically acting with endogenous NO to increase sGC activity[304]. sGC activators, such as cinaciguat, bind to the heme pocket and provide an additive effect to NO by replicating the NO-heme complex needed for activation[259]. Decreased NO bioavailability and perturbed sGC signaling has been discovered in many diseases such as cardiovascular disease, heart failure, ischemia and reperfusion injury (I/R), stroke, and pulmonary and systemic hypertension[261,305-308]. In the kidney specifically, a role for sGC signaling has been suggested and shown in models of chronic kidney disease, anti-thy1 glomerulonephritis, 5/6 nephrectomy, type-1 and type-2 diabetes, and I/R-induced AKI.

Many studies, have shown and supported the role for mitochondrial dysfunction in the pathogenesis of I/R-induced AKI[61,133,154,309,310]. Moreover, some studies have suggested that cGMP can regulate MB[71,72,311]. There are two ways of directly sustaining cGMP production. The first is to activate sGC and the second is to inhibit the degradation of cGMP by targeting PDEs. PDE5, PDE6, and PDE9 have a higher affinity for targeting cGMP than cAMP for degradation[266]. We have previously shown *in vivo*, that targeting cGMP production by administering a PDE5 inhibitor, Sildenafil, attenuated renal dysfunction and the decrease in MB[73]. However, the role of sGC in regulating MB after

I/R is still under investigation. Here we characterize sGC signaling in the early phase of I/R *in vivo* by focusing on the first 24 hours of injury, the peak of maximal renal dysfunction.

Material and Methods

Naïve mice studies

Mice were sacrificed at 8 weeks of age and tissues were harvested and immediately flash frozen.

Ischemia-reperfusion (I/R)-induced AKI mouse model

Mice were subjected to bilateral ischemia where both renal pedicles were clamped for 18 min at 37°C and then removed to reintroduce blood flow as previously described.[155] Sham mice were operated on in the same manner except for no clamping. Mice were sacrificed 24 h after injury and blood was collected via retro-orbital bleed. All tissues were flash frozen immediately.

In vivo sGC activity

When sacrificing mice, approximately half of the renal cortex is immediately isolated and put in 1mL of ice cold PBS and vortexed for 1 minute. The cold PBS is removed and the tissue is submerged in 500uL of lysis buffer including 50mM Tris-HCl, 1mM EDTA, 1mM dithiothrietol and 2 mM phenylmethyl sulphonyl fluoride (PMSF) at pH 7.6. Tissue was homogenized using a dounce homogenizer and the mixture was centrifuged at 20,000g for 20 min at 4C. Supernatants were collected and protein was measured. Reaction was prepared as previously described.[250] Briefly, 50ug of protein was incubated with 100uL of reaction mixture containing 50mM Tris-HCl at pH:7.5, 7.5mM creatine phosphate, 0.2 mg/mL creatine phosphokinase, 1mM GTP, 4 mM MgCl2, 0.5 mM IBMX, and 1mM L-NAME with either 10uM riociguat, 10uM cinaciguat, 200nM mahma

nonoate at 37C. The reaction was terminated with the addition of 400uL of .05M of HCl. Products were flash frozen and stored at -80C until ready to measure cGMP by cGMP Elisa kit from Cayman Chemical.

qRT-PCR

RNA was isolated from renal cortical tissue using Trizol (Life Technologies). cDNA was produced using the iSCRIPT Advanced cDNA Synthesis Kit (Biorad) according to the manufacturer's protocol. SsoAdvanced Universal SYBR Green Supermix reagent (BioRad) was used with the generated cDNA according to manufacturer's protocol. mRNA expression was determined using a 2-triangle triangle CT method where mouse actin RNA was used for normalization.

Immunoblot Analysis

Half of frozen renal cortical tissue was isolated and put in RIPA buffer containing 50 mM Tris-HCl, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, pH 7.4 with phosphatase inhibitors (1:100), 1mM sodium orthovanadate, and 1 mM sodium fluoride. Tissue was pulverized using a hand held homogenizer and sonicated for approximately 10 seconds. Tissue was centrifuged at 14,000 g for 15 min at 4C. Supernatants were removed and protein was measured using a BCA assay.

Equal protein was loaded onto 4-15% SDS page gels and separated by gel electrophoresis. Protein was transferred onto nitrocellulose membranes and blocked in 5% milk or 5% bovine serum albumin dissolved in TBST. Membranes were submerged

in primary antibodies overnight with rotation. The next day membranes were washed in TBST 3 times for 5 minutes each and were rotated in appropriate horseradish peroxidase–conjugated secondary antibody. Membranes were visualized using enhanced chemiluminescence (Thermo Scientific) and the GE ImageQuant LAS4000 (GE Life Sciences). Optical density was determined using the ImageJ software from NIH.

Measuring tissue cGMP

Renal Cortical tissue was isolated from one whole kidney and cGMP was measured according to manufacturer's protocol (Cayman Chemical).

Results

sGC activity in naïve mice

We sought to examine sGC activity in the kidneys of naïve mice. We measured sGC activity in fresh renal cortex homogenates as previously described[250] and determined linearity of the reaction in terms of homogenate protein content and reaction time. Frozen homogenates exhibited a marked reduction in sGC activity (data not shown). The NOS inhibitor L-NAME (1mM) was included in all to assays to prevent NO formation. cGMP formation was linear between 0.5 and 2 μg/μl and between 5 and 15 min (Fig. 2.1A). Using a homogenate protein concentration of 1 μg/μl and a 5 min reaction time, we added the NO donor, mahma nonoate (200nM) and observed a 3.4 fold increase in sGC activity compared to vehicle control (Figure 2.1B).

To determine the effects of sGC activators and stimulators on sGC activity, we added riociguat (10 μM) or cinaciguat (10 μM), and observed 12-fold and 18-fold increases respectively (Figure 2.1B). We expected to see a robust increase in sGC activity with the addition of riociguat, because the reduced form of sGC predominates in naïve mice. Alternatively, cinaciguat targets only the oxidized/hemeless state of sGC, a small pool of oxidized sGC that exists in naïve mice[312]. Taken together, these results validate our assay for measuring sGC activity in the renal cortex and reveals marked increases in cGMP in responses to riociguat and cinaciguat.

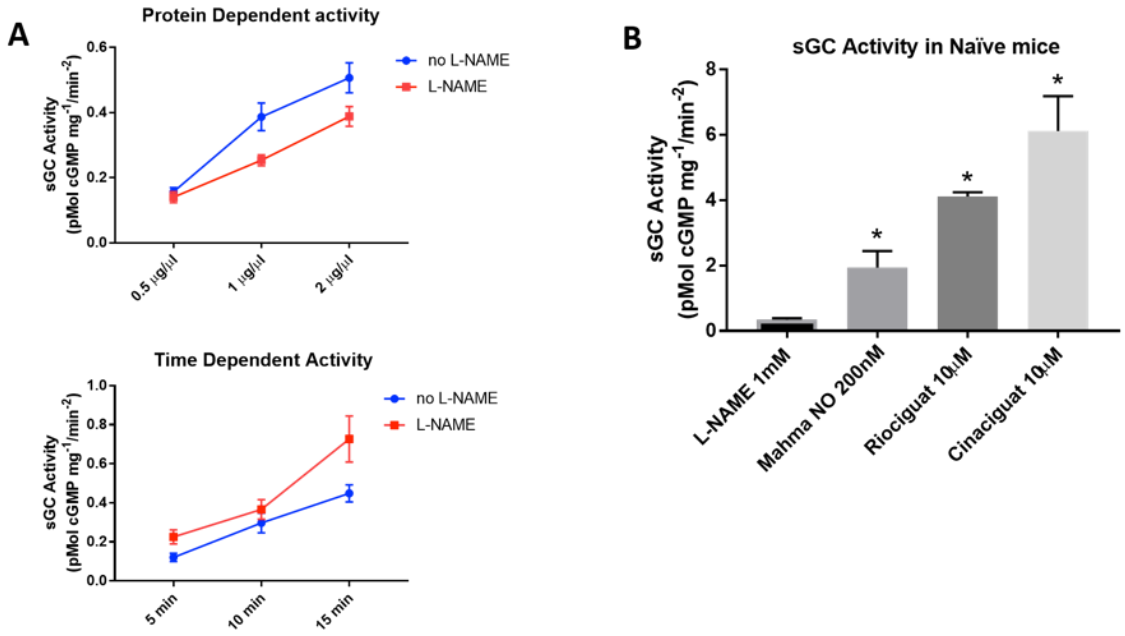

Figure 2.1: sGC activity in naïve mice. Naïve mice at 8-10 weeks were sacrificed and renal cortical tissue was immediately isolated for determination of sGC activity. A) sGC activity determined in naïve mice on a protein and time dependent scale. B) sGC activity in naïve mice. Homogenates are dosed with 200nM mahma nonoate, 10uM riociguat, and 10uM cinaciguat. N=11-12. * represents significance compared to L-NAME 1mM ($p<0.05$)

sGC expression after I/R

To confirm that mice subjected to I/R exhibited renal dysfunction at 24 h, serum creatinine was determined. Serum creatinine increased 11-fold to 1.8 mg/dL in I/R mice compared to Sham mice and renal cortical protein expression of kidney injury marker-1 (KIM-1) and NGAL was observed in I/R mice (Figure 2.2A).

To begin characterizing sGC signaling after I/R, we measured renal cortical protein and mRNA expression of the alpha 1 and beta 1 subunits 24 h after injury. There were no significant changes in protein expression of the alpha 1 and beta 1 subunits between sham and I/R mice (Figure 2.2C,E). Only mRNA expression for the alpha 1 subunit was significantly decreased in I/R mice compared to sham mice (Figure 2.2B).

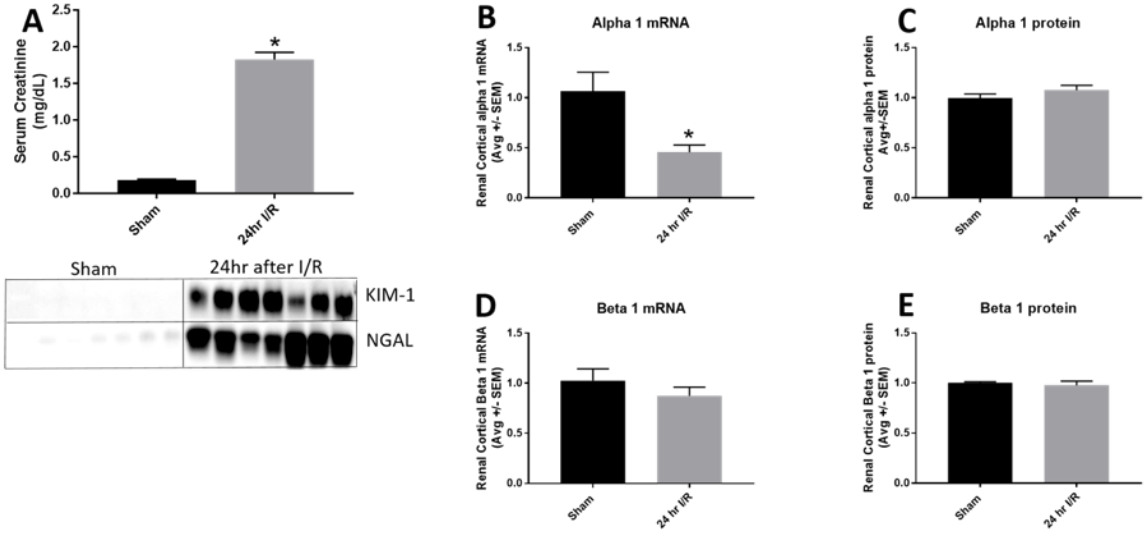

Figure 2.2: sGC expression after I/R A) Serum Creatinine of I/R mice compared to Sham in mg/dL. Representative blot of KIM-1 and NGAL. B and D) mRNA expression of Alpha 1 and Beta 1 subunits following I/R. C and E) densitometry analysis of alpha 1 and beta 1 subunits following I/R. Data are represented as mean S.E., N=6-7. * represents significance compared to Sham ($p<0.05$)

NO-responsive/reduced sGC decreases after I/R

We then detected sGC activity in the renal cortex 24h after I/R in the presence of riociguat and cinaciguat (Figure 2.3A). We found that basal sGC activity in I/R homogenates (I/R+LN) was significantly increased by 1.4 fold compared to Sham homogenates (Sham+LN). Responses to riociguat and cinaciguat were all significant compared to Sham+LN.

We wanted to examine this data in a different manner to assess the individual changes between mice. sGC activity in Sham and I/R homogenates treated with riociguat and cinaciguat were normalized to their respective basal activities (Figure 2.3B). For example the value for I/R+cinaciguat was normalized back to the I/R+LN value for that mouse. sGC activity in Sham homogenates dosed with riociguat and cinaciguat were increased 18.3-fold and 21.1-fold compared to Sham+LN, respectively. However, sGC activity in I/R homogenates also dosed with riociguat and cinaciguat, showed a 9.5-fold and 17-fold increase compared to I/R+LN respectively. Using this approach, we observed a significant decrease in sGC activity in I/R+riociguat compared to Sham+riociguat. There was no difference between Sham+cinaciguat and I/R+cinaciguat groups. This indicates a decreased presence of the NO-responsive/reduced sGC after I/R.

Although we have found an increase in sGC activity after I/R, we measured cGMP in the renal cortex of sham and I/R mice (Figure 2.3D). We observed a significant decrease in cGMP 24 h after I/R compared to Sham. The decrease in the reduced state of sGC is consistent with the decrease in renal cGMP. Oxidized sGC retains basal activity, however, it does not respond to NO resulting in a decrease in renal cGMP, as we have shown.

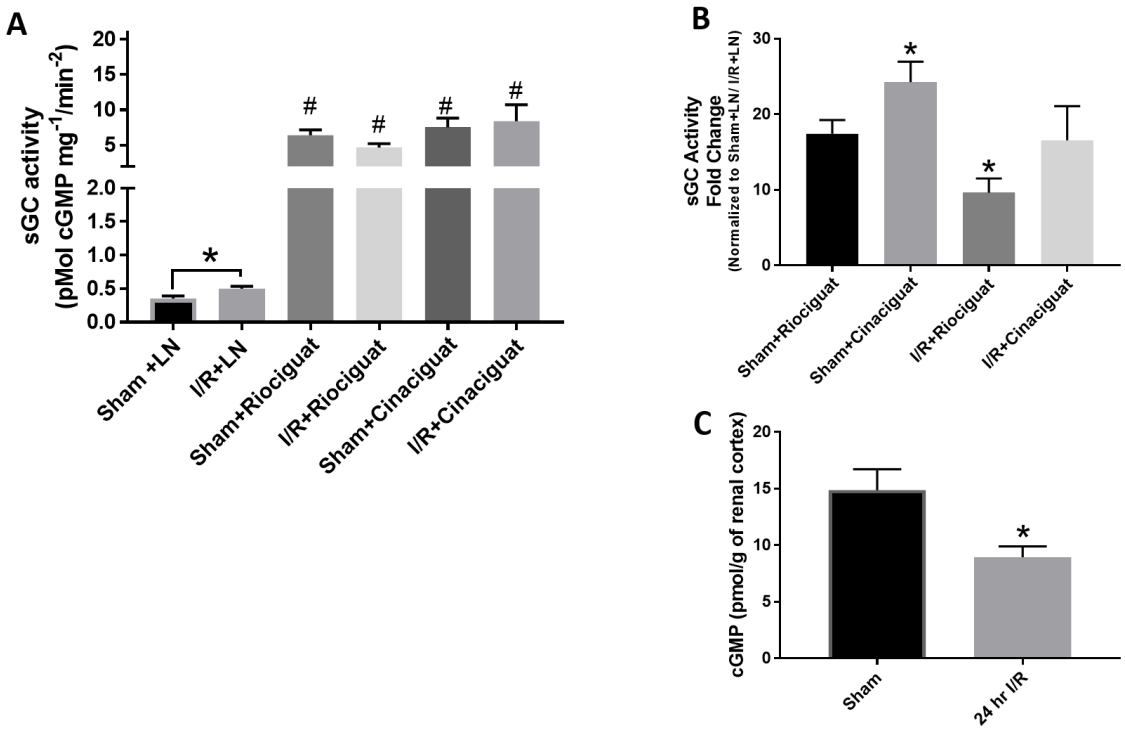

Figure 2.3: NO-responsive/reduced sGC decreases after I/R A) sGC activity 24hr after I/R. B) Normalization of sGC activity to their respective L-NAME controls. * represents significance compared to Sham+riociguat C) Renal cortical cGMP 24h after I/R. Data are represented as mean S.E., N=6-12. * represents significance compared to Sham+LN/Sham for 3a ($p<0.05$)

PKG activation increases after I/R

An increase in cGMP production can activate cGMP-gated ion channels, protein kinase G (PKG), and phosphodiesterases. At 24 h after I/R, PKG1α protein and mRNA were not affected (Figure 2.4C-D). Here we show that an increase in sGC activity activates PKG, 24 h after I/R (Figure 2.4A-B). We determined the activation of PKG by measuring the phosphorylation of vasodilator-stimulated-phosphoprotein, VASP, a target of PKG[295]. We found an increase in the phosphorylation of VASP and in total VASP after I/R as shown in Figure 2.4A-B.

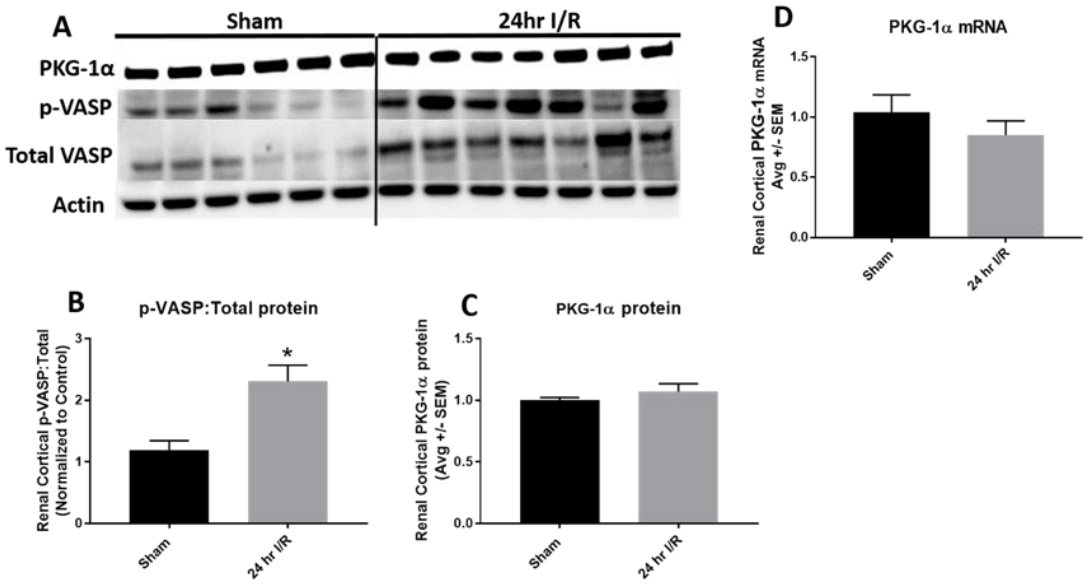

Figure 2.4: PKG activation increases after I/R A) Representative blot of PKG-1a, p-VASP, Total VASP, and Actin showing Sham and I/R. B) Densitometry analysis of p-VASP. C) Densitometry analysis of PKG-1a. D) mRNA expression of PKG-1a following I/R. Data are represented as mean S.E., N=6-7. * represents significance compared to Sham for ($p<0.05$).

PKG-associated downstream targets increase after I/R

Numerous studies have shown a trend between PKG activation and an increase in p38 and/or ERK1/2 depending on the cell type[299]. We measured phosphorylation of p38 and found that it was increased by 2.2-fold in I/R compared to Sham mice (Figure 2.5A-B). We measured ERK1/2 phosphorylation and found a 3-fold increase in I/R compared to Sham (Figure 2.5A and C).

Previous reports from this lab have elucidated the role of ERK1/2 phosphorylation in our model of I/R-induced AKI[313,314]. We reported that 3 h after I/R, ERK1/2 phosphorylation increases, subsequently increasing FOXO1 phosphorylation, a direct target of ERK1/2. Interestingly, phosphorylated p38 also targets FOXO1[315]. We measured FOXO1 phosphorylation and found a significant decrease in p-FOXO1 at 24hr after I/R (Figure 2.5D).

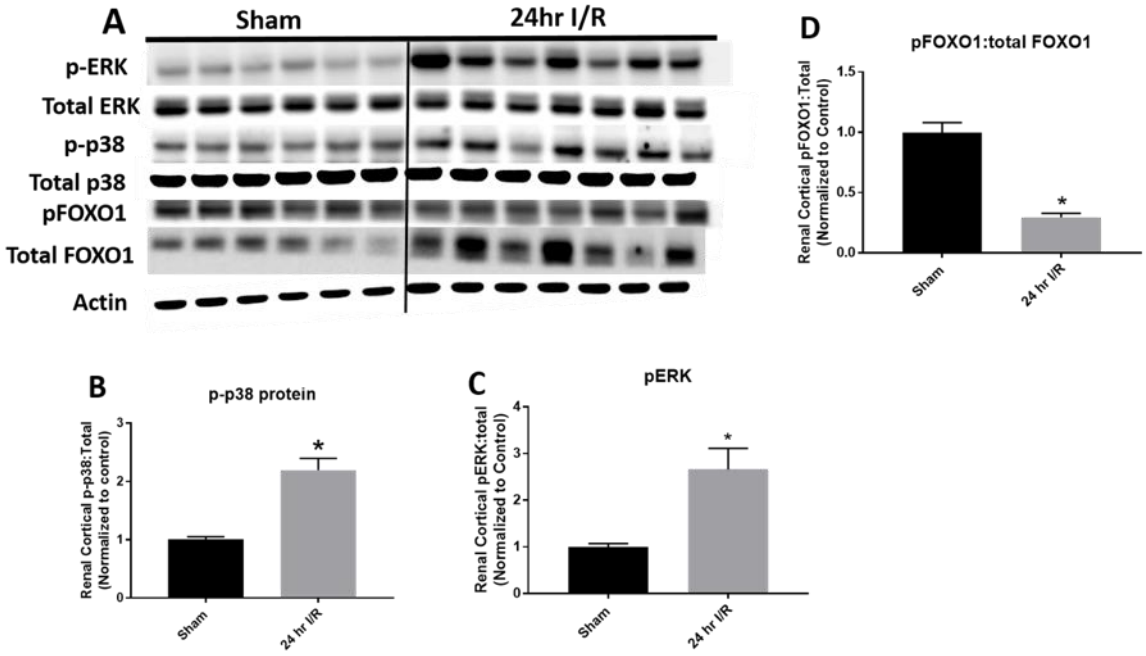

Figure 2.5: PKG-associated downstream targets increase after I/R A) Representative blot of p-ERK, Total ERK, p-P38, Total P38, pFOXO1, Total FOXO1, Actin. B) Densitometry analysis of p-P38 following I/R. C) Densitometry analysis of pERK following I/R. D) Densitometry analysis of p-FOXO1 following I/R. Data are represented as mean S.E., N=6-7. * represents significance compared to Sham for ($p<0.05$).

MB is decreased 24 h after I/R

We previously reported observing a decrease in MB 24 h after I/R. mRNA and protein expression of mitochondrial proteins such as NDUFB8, COX1, and ATP Synthase β were decreased after 24 h after I/R[155]. We confirmed the decrease in MB in our model by measuring PGC-1α. We found a significant decrease in mRNA expression (Figure 2.6a) and protein expression of PGC-1α after I/R (Figure 2.6b).

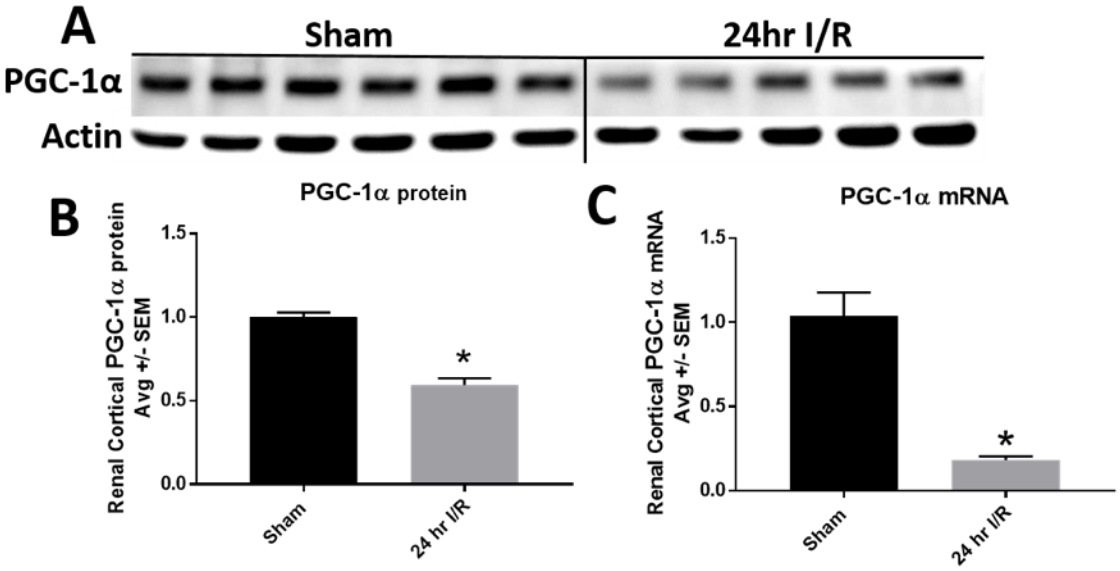

Figure 2.6: MB is decreased 24 h after I/R A) Representative blot of PGC-1α and Actin. B) Densitometry analysis of PGC-1α following I/R. C) mRNA expression of P PGC-1α. Data are represented as mean S.E., N=6-7. * represents significance compared to Sham for ($p<0.05$).

The effect of Trametinib on sGC/cGMP/PKG pathway in I/R

Based on our previous studies concerning the role of ERK1/2 in the early phase of I/R and as a potential mediator between PKG activation and decreased MB, we explored the effect of ERK1/2 inhibition on sGC signaling. To inhibit ERK1/2, we administered an inhibitor targeting a protein upstream of ERK1/2 called MEK1/2. Our previous studies show that administration of trametinib, a MEK1/2 inhibitor, effectively attenuated renal dysfunction by attenuating serum creatinine levels as well as MB at 3 h. We focused on 3 h after injury, as events at this point play a major role in the resulting maximal dysfunction at 24 h. We measured the phosphorylation of eNOS and PKG activation at 3 h with and without trametinib in Figure 2.7. We found a significant increase in phosphorylation of eNOS at ser1177 at 3 h after I/R and trametinib attenuated this increase (Figure 2.7A). At 3 h, phosphorylation of VASP is trending upwards and trametinib did not have a significant effect on PKG activation (Figure 2.7B).

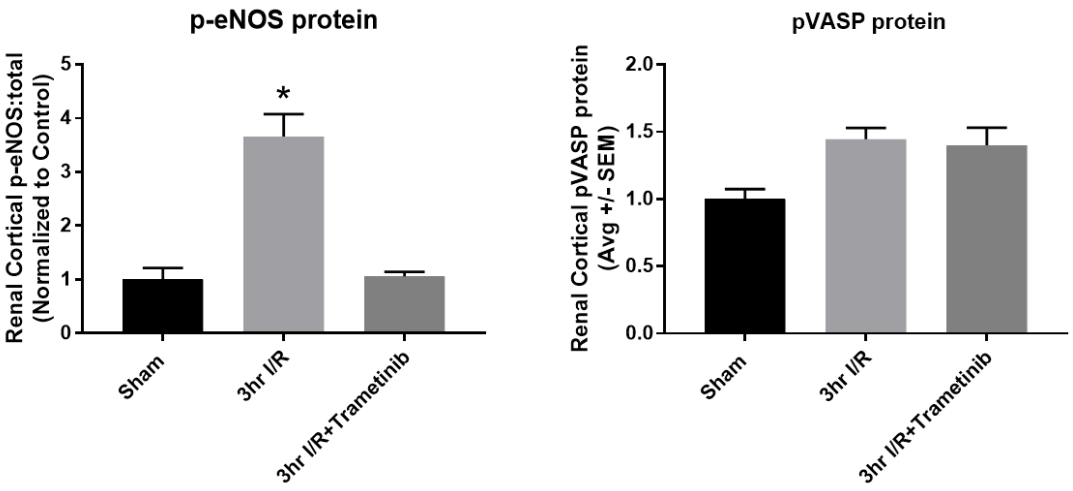

Figure 2.7: The effect of Trametinib on sGC/cGMP/PKG pathway in I/R A) Densitometry of p-eNOS following 3 h after I/R. B) Densitometry of p-VASP following 3 h after I/R. Data are represented as mean S.E., N=3-4. * represents significance as determined by one-way ANOVA ($p<0.05$).

Discussion

Previously in our laboratory, we have shown a persistent decline in mitochondrial function after I/R. PGC-1α, the master regulator of mitochondrial biogenesis (MB) is decreased 24 h after I/R as well as other mitochondrial proteins such as NDUFB8, ATP synthase B, and cytochrome c oxidase subunit 1 (COX1)[155]. Although we have identified several mechanisms that play a role in attenuating MB after I/R, the role of sGC is still under investigation.

In naïve mice, sGC is predominantly in its reduced state, meaning the heme moiety is in its ferrous form. The ferrous form allows for proper binding of NO and subsequent cGMP production. To be able to measure the reduced form of sGC, we optimized an sGC enzyme assay from Thoonen et al, for the kidney[250]. After we showed linearity of our sGC assay at a protein and time dependent scale, in Figure 2.1b we show a robust response with mahma nonoate, the NO donor, and riociguat, the sGC stimulator that targets the reduced heme in sGC. The oxidation of the ferrous heme to ferric renders sGC less active unless treated with cinaciguat, the sGC activator that binds to the heme pocket sGC. However, oxidized sGC with no pharmacological administration still retains a certain level of basal sGC activity[250,316]. Studies have shown the presence of oxidized sGC in primary cultures of ovine fetal PASMCs (pulmonary arterial smooth muscle cells) by treating them with cinaciguat[316]. Thoonen et al, also showed the presence of sGC activity in apo-sGC mice compared to control mice[250]. The apo-sGC mice carry a H105F point mutation in the catalytic site of the beta 1 subunit, allowing for improper ligation of the heme moiety in the catalytic site. With this mutation, although the homogenates from

apo-sGC mice did not respond to an NO donor, these homogenates still had basal level sGC activity[250]. These studies support the existence for basal level activity of oxidized/heme-free sGC. Here in Figure 2.1b, we show a robust response in sGC activity when homogenates from naïve mice are dosed with cinaciguat. Our results are consistent with previous literature regarding the presence of oxidized sGC in naïve mice.

In terms of I/R, other models of organ dysfunction such as cardiac I/R have shown that sGC can become oxidized and potentially heme free subjecting sGC for degradation[257,317,318]. In Figure 2.2, we found no change in the protein expression of the sGC subunits and therefore sought to measure sGC activity. In Figure 2.3, we show a decreasing presence of the reduced form of sGC after I/R, consistent with other models of I/R. The decrease in renal cortical tissue cGMP could be a result of the reduced form of sGC after I/R. The increase in basal sGC activity is not enough to be reflected at the tissue level, however seems sufficient enough to activate PKG, as shown in Figure 2.4. Although phosphorylation of VASP at ser239, a marker for cGMP specific activation of PKG, increases after I/R, interestingly we also found an increase in total VASP. This increase in total VASP could be due to the severity of renal injury, shown in Figure 2.2a. VASP is associated with regulating actin cytoskeleton and migration of certain types of cells, including renal cell types[319,320]. VASP is also associated with endothelial cell integrity in the kidney. Recent studies have also suggested an important role for VASP in renal injury[321]. VASP deficient mice subjected to I/R, show decreased long-term progression of nephrotoxic nephritis compared to wild type[322]. The increase in total

VASP can be a marker for injury and endothelial dysfunction therefore showing that the increase in PKG activation certainly plays a role in renal dysfunction after I/R[321].

The signaling pathway from increased PKG activation to decreased MB in I/R is still under investigation. p38 MAP Kinase and ERK1/2 MAP kinase are two regulators of PGC-1α in the kidney. Previous studies in our lab have elucidated the role for ERK1/2 in the regulation of MB after I/R[313,314]. We have also shown the importance of p38 in renal proximal tubular epithelial cell proliferation[323]. Both p38 and ERK1/2 also happen to be PKG-associated downstream targets. There is an abundance of literature regarding the correlation between PKG activation and downstream regulation of p38 and ERK1/2 in other models of I/R as well as the kidney[266,268,299,324,325]. We sought to explore the role of p38 and ERK1/2 as potential mediators between PKG activation and the down regulation of MB.

We found an increase in the phosphorylation of p38 and ERK1/2 as shown in Figure 2.5. Previously we have shown that ERK1/2 can regulate PGC-1a transcription and subsequently MB, by phosphorylation of FOXO1, a transcription factor and a target of p38 as well[315]. Phosphorylation of ERK1/2 causes a phosphorylation of FOXO1, inhibiting the translocation of FOXO1 inside the nucleus. This causes a decrease in PGC-1α mRNA and therefore MB. We sought to explore if ERK1/2/FOXO1 signaling was a mediator between PKG activation and PGC-1α mRNA. We measured the phosphorylation of FOXO1 and found that it was significantly decreased (Figure 2.5E). The phosphorylation of FOXO1 by ERK1/2 may not be necessary at 24 h since PGC-1α

protein and mRNA expression is decreased by this time point (Figure 2.6). These data show that ERK1/2 is not regulating MB through the phosphorylation of FOXO1 and this finding is unrelated to PKG activation.

However, since the phosphorylation of FOXO1 may not be necessary for regulating PGC-1α transcription at 24 h, the role of ERK1/2 could still be a mediator between PKG activation and decreased MB. Therefore, we explored the effect of trametinib, a MEK 1/2 inhibitor, on sGC signaling at 3 h in Figure 2.7. We focused on this time point because previous studies have shown that pretreatment with trametinib before I/R can attenuate serum creatinine within 3 h after I/R. Trametinib attenuated the increase in phosphorylated eNOS. The increase in phosphorylated eNOS could be producing nitric oxide that is toxic therefore sustaining injury. Trametinib did not attenuate the increase in PKG activation suggesting that trametinib may play a role in PKG activation independent of I/R.

It is possible that p38 may play a role in suppressing MB however, we need to do further *in vivo* studies to elucidate the role and necessity of p38 after I/R. This study provides another perspective to the role of sGC in I/R. Further investigation is needed to determine if ERK1/2 is truly a mediator between PKG activation and decreased MB. Thus far, we have provided sufficient evidence hinting at ERK1/2 involvement in positively regulating sGC signaling and therefore playing a role preventing the suppression of MB 24 h after I/R.

Chapter Three: Elucidation of cGMP-dependent induction of Mitochondrial Biogenesis through Protein Kinase G and p38 MAPK

Introduction

Soluble Guanylyl Cyclase (sGC) produces cGMP by converting GTP in the presence of nitric oxide (NO). cGMP can bind to cGMP gated ion channels, phosphodiesterases, and protein kinase G (PKG). cGMP plays a role in a variety of processes in the cell besides vasodilation, platelet aggregation, and smooth muscle vasorelaxation. Nisoli and group proposed that nitric oxide induces mitochondrial biogenesis through cGMP[71,311]. In our laboratory, we have shown mitochondrial respiration increase significantly in renal proximal tubule cells (RPTC) with 10μM b-Br-cGMP compared to cAMP suggesting that cGMP is responsible for inducing MB rather than cAMP[73]. Moreover, when RPTC are dosed with 8-Br-cGMP for 24 h, mRNA for several mitochondrial proteins increases as well as the master regulator of MB, PGC-1α increased[73].

Here, we have elucidated a pathway from cGMP to this increase in PGC-1α mRNA by focusing on events that may occur with in an hour of exposure to 8-Br-cGMP. Several studies have supported the role for PKG involvement in mitochondrial function. PKG is a serine/threonine kinase and exists in two forms, PKG1 and PKG2. In renal tubular cells, PKG1 activity and expression decreased when exposed to cisplatin[324]. Increasing PKG1 activity protected mitochondrial function, preventing cell apoptosis induced by cisplatin[324]. In an *in vitro* model for diabetes, adipocytes exposed to lipoamide were found to also induce MB through PKG providing a therapeutic target[326]. It was also shown in brown adipose tissue, that PKG mediates the effects of cGMP by inducing MB and increasing UCP-1[327]. Based on this literature, we chose to focus on PKG1, as the main target of cGMP, after exposing RPTC to 8-Br-cGMP.

Although there are many direct targets of PKG, including subunits of a variety of ion channels, there are few downstream associated targets such as ERK1/2, GSK3β, Akt, and p38[328]. The exact mechanism from PKG to these targets is unknown and still under investigation. We chose to focus on p38 for its role in regulating PGC-1α expression at the transcriptional and posttranslational level. Moreover, nitric oxide donors can induce p38 activation[268,325,329]. In brown adipose tissue, naturetic peptides activate Guanylyl Cyclase (GC) resulting in activated PKG[330,331]. Activated PKG leads to the phosphorylation of p38[330]. Phosphorylated p38 can phosphorylate PGC-1α increasing PGC-1α transcription. We sought to explore if this mechanism exists in RTPC.

Material and Methods

In vitro Studies:

Female New Zealand White rabbits (approximately 2Kg) were purchased from Charles River (Oakwood, MI/Canada). Renal Proximal tubule cells (RPTC) were isolated from kidneys using the iron oxide perfusion method. Cells were plated and grown on 35 mm tissue culture dishes in conditions that are similar to physiological conditions *in vivo,* as described previously[332]. The culture medium consisted of a 1:1 mixture of Dulbecco's modified Eagle's medium/F-12 (without glucose, phenol red, or sodium pyruvate) with 15mM HEPES buffer, 2.5 mM L-glutamine, 1 uM pyridoxine HCl, 15 mM sodium bicarbonate, and 6 mM lactate. Hydrocortisone (50 nM), selenium (5 ng/ml), human transferrin (5 ug/ml), bovine insulin (10 nM), and L-ascorbic acid-2-phosphate (50 uM) were added to fresh culture medium. Only confluent RPTC were dosed with either compound or DMSO. The vehicle control for the inhibitor studies were dosed separately when dosing with inhibitors of PKG and p38.

Subcellular Fractionation:

Cells were harvested in 300uL of sucrose isolation (ISO) buffer containing 250 mM sucrose, 1 mM EGTA, 10 mM HEPES, and 1 mg/ml fatty acid free BSA at a pH of 7.4. Cells were homogenized using a dounce homogenizer and were then spun at 700 g for 5 min. The supernatant is the cytosolic fraction was stored in another centrifuge tube with phosphatase inhibitors (1:100), 1mM sodium orthovanadate, and 1 mM sodium fluoride and concentrated Triton X-100 and SDS at 4%. The pellet was washed twice in ISO buffer and spun down at 1,000 g for 5 min each time. The pellet was then re-suspended in

200uL of RIPA buffer, phosphatase inhibitors (1:100), 1mM sodium orthovanadate, and 1 mM sodium fluoride.

Immunoblot Analysis

RTPC were harvested in 200uL RIPA buffer containing 50 mM Tris-HCl, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, pH 7.4 with phosphatase inhibitors (1:100), 1mM sodium orthovanadate, and 1 mM sodium fluoride. Cells were sonicated for approximately 10 seconds. Cells were centrifuged at 7,500 g for 5 min at 4C. Supernatants were removed and protein was measured using a BCA assay.

Equal protein was loaded onto 4-15% SDS page gels and separated by gel electrophoresis. Protein was transferred onto nitrocellulose membranes and blocked in 5% milk or 5% bovine serum albumin dissolved in TBST. Membranes were submerged in primary antibodies overnight with rotation. The next day membranes were washed in TBST 3 times for 5 minutes each and were rotated in appropriate horseradish peroxidase–conjugated secondary antibody. Membranes were visualized using enhanced chemiluminescence (Thermo Scientific) and the GE ImageQuant LAS4000 (GE Life Sciences). Optical density was determined using the ImageJ software from NIH.

qRT-PCR

Cells were harvested in 400uL of Trizol (Life Technologies). cDNA was produced using the iSCRIPT Advanced cDNA Synthesis Kit (Biorad) according to the manufacturer's protocol. SsoAdvanced Universal SYBR Green Supermix reagent (BioRad) was used with the generated cDNA according to manufacturer's protocol. mRNA expression was

determined using a 2-triangle triangle CT method where rabbit tubulin RNA was used for normalization.

Results

8-Br-cGMP activates PKG but does not affect PGC-1α protein expression

To begin elucidating the pathway from cGMP production to PGC-1α expression, RTPC were treated with a cGMP analog, 8-Br-cGMP (10μM) for 1 h. Several studies have suggested that Protein Kinase G, PKG, could play a role in the regulation of mitochondrial biogenesis. Here we measured PKG activation by measuring the phosphorylation of vasodilator-stimulated phosphoprotein (VASP) at site serine 239 (Figure 3.1A, 3.1C). We found a 1.9-fold increase in the phosphorylation of VASP compared to vehicle control (Figure 3.1C). We also treated RPTC with 10μM riociguat, an sGC stimulator that specifically targets the reduced in sGC. We found a 1.2-fold increase in the phosphorylation of VASP compared to control.

We pretreated RPTC with 100nM KT5823 (KT), a PKG inhibitor, for 30 min and then treated with DMSO vehicle, 10μM 8-Br-cGMP, or 10μM riociguat for 1 h. These particular sets of experiments have their own vehicle control to take into account of additional DMSO due to pretreatment and to eliminate any effect caused by DMSO. Phosphorylation of VASP was decreased 30% in the KT+V treated group. In the KT+cGMP treated group, phosphorylation of VASP also decreased however, this decrease is not significant if compared to 8-Br-cGMP. KT did not affect the phosphorylation of VASP after the addition of riociguat. At 1 h, we then measured PGC-1α protein expression in all treatment groups (Figure 3.1B). We found no change in protein expression of RPTC dosed with 8-Br-cGMP, riociguat, KT+V, KT+cGMP, and KT+riociguat compared to vehicle control.

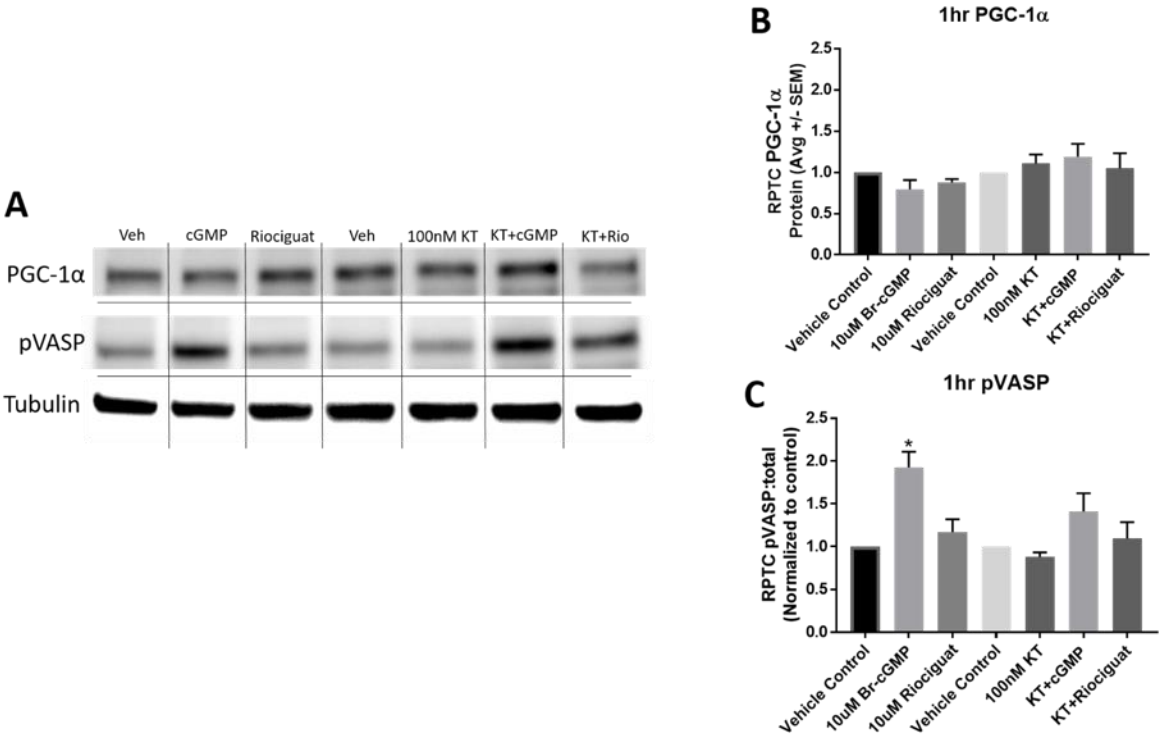

Figure 3.1: 8-Br-cGMP activates PKG but does not affect PGC-1α protein expression A) Representative blot for PGC-1α, pVASP, and Tubulin after treatment. B) Densitometry analysis for PGC-1α protein. C) Densitometry analysis for pVASP protein. Data are represented as mean S.E., N=7-8. * represents significance compared to Vehicle ($p<0.05$).

PGC-1α is phosphorylated and is located in the nucleus in the presence of 8-Br-cGMP

Since we detected no change in PGC-1α protein expression at 1 h, we sought to measure the phosphorylation of PGC-1α, an event that may be possible within 1 h of dosing. Moreover, we were interested in knowing the location of phosphorylated PGC-1α. Phosphorylation of PGC-1α at serine and threonine sites by p38 can increase the stability of PGC-1α and sustain transcription of PGC-1α[62]. We treated RPTC with 8-Br-cGMP, riociguat, and KT, similar to the dosing regimen in Figure 3.1. We first subjected RPTC to subcellular fractionation and tested for purity of nuclear and cytosolic fractions by measuring alpha tubulin, a cytosolic marker, and Lamin B, a nuclear marker (Figure 3.2A). Secondly, we immunoprecipitated PGC-1α and blotted for phosphorylated serine and threonine residues in both the nuclear and cytosolic fractions (Figure 3.2B-C). We found a significant increase in phosphorylated PGC-1α in the nuclear fraction when dosed with 8-Br-cGMP by 1.64 fold (Figure 3.2D). We found that treatment with KT inhibited phosphorylation of PGC-1α in the nucleus. We did not find any significant changes in the phosphorylation of PGC-1α in the cytosolic fraction compared to vehicle control, although it seems phosphorylation of PGC-1α is trending upwards when RPTC are pretreated with KT (Figure 3.2E).

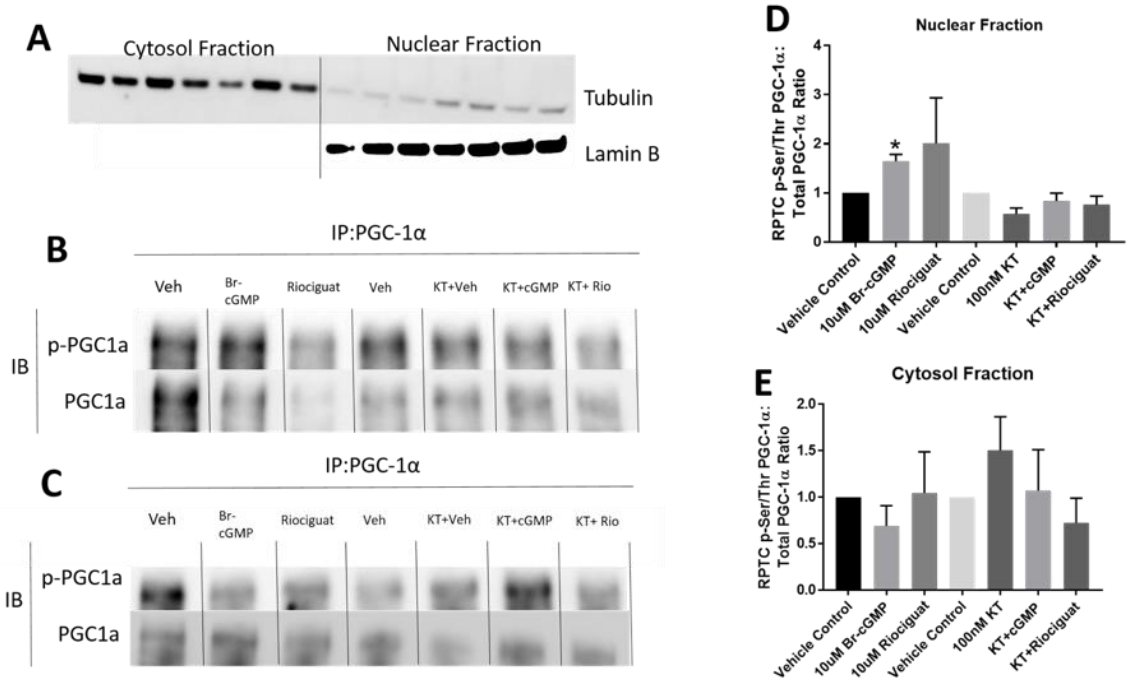

Figure 3.2: PGC-1α is phosphorylated and is located in the nucleus in the presence of 8-Br-cGMP A) Representative blot of Tubulin and Lamin B showing purity of Cytosol and Nuclear fractions. B and C) phosphorylated serine and threonine residues were measured following immunoprecipitation of PGC-1α by immunoblot analysis after 30 min treatment with DMSO or 100nM KT5823 followed by exposure to DMSO, 8-Br-cGMP, and Riociguat in cytosol and nuclear fractions. Total PGC-1α expression was measured after immunoprecipitation. D and E) Densitometry analysis for phosphorylated serine and threonine residue in the cytosol and nuclear fractions. Data are represented as mean S.E., N=3-4. * represents significance compared to Vehicle control ($p<0.05$).

KT5823 inhibits p38 phosphorylation

Due to our results in Figure 3.2, we hypothesized that p38 is the mediator between PKG activation and phosphorylation of PGC-1α. Many studies have suggested a role for p38 in the regulation of MB when NO donors are administered in vitro. Previously in this laboratory we have also shown that p38 is necessary for RPTC proliferation and that p38 may play a role in recovery of RPTC when exposed to oxidant injury[323,333]. p38 can also phosphorylate PGC-1α at Threonine 298, Threonine 262, and Serine 265 as mentioned above and can increase the stability of PGC-1α in brown adipose tissue[62]. To elucidate this signaling, we measured p38 phosphorylation (p-p38) in the presence and absence of 100nM KT (Figure 3.3). We that found that 8-Br-cGMP increased p-p38 by 1.5 fold. Riociguat also significantly increased p-p38. KT inhibited the phosphorylation of p38 when dosed with vehicle, 8-Br-cGMP and riociguat.

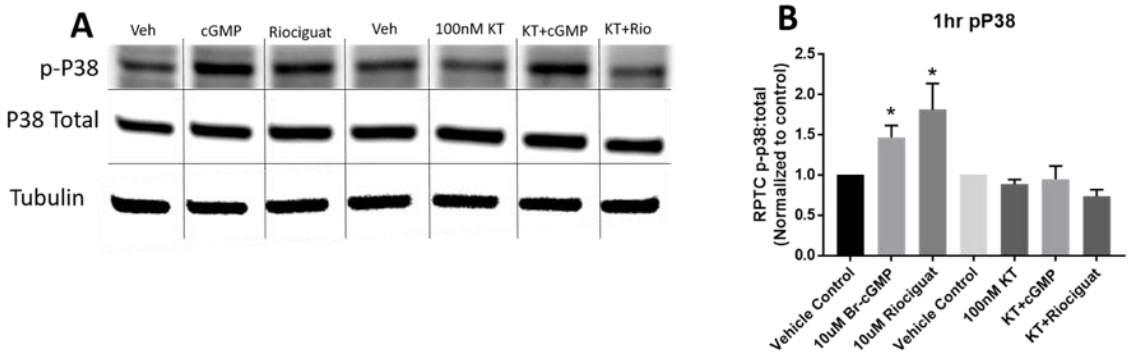

Figure 3.3: KT5823 inhibits P38 phosphorylation A) Representative blot for p-P38, p38 total, and Tubulin. B) Densitometry analysis for p-p38 protein. Data are represented as mean S.E., N=7-8. * represents significance compared to Vehicle ($p<0.05$).

Inhibition of p38, inhibits PGC-1α phosphorylation at serine and threonine sites

Although p38 is downstream of PKG activation based on our results, we sought to explore whether p38 is phosphorylating PGC-1α. We pretreated RPTC, similar to the KT5823 experiments, for 30 min with an inhibitor of p38, L-skepinone, at 100nM and then treated with vehicle, 10μM 8-Br-cGMP, and 10μM riociguat for 1 h and 2 h. We wanted to first validate inhibition of the phosphorylation of p38 at 1 h and at 2 h (Figure 3.4A-B). We found that administration of 100nM of L-skepinone (LS) successfully inhibited the phosphorylation of p38 when dosed with 8-Br-cGMP and riociguat at both 1 h and 2 h (Figure 3.4C-D). We then measured phosphorylation of PGC-1α with L-skepinone (Figure 3.4E-F). We found no significant change between L-Skepinone (LS) treated groups (LS+Vehicle, LS+cGMP, LS+riociguat) and vehicle. This indicates that L-Skepinone inhibited the phosphorylation of PGC-1α.

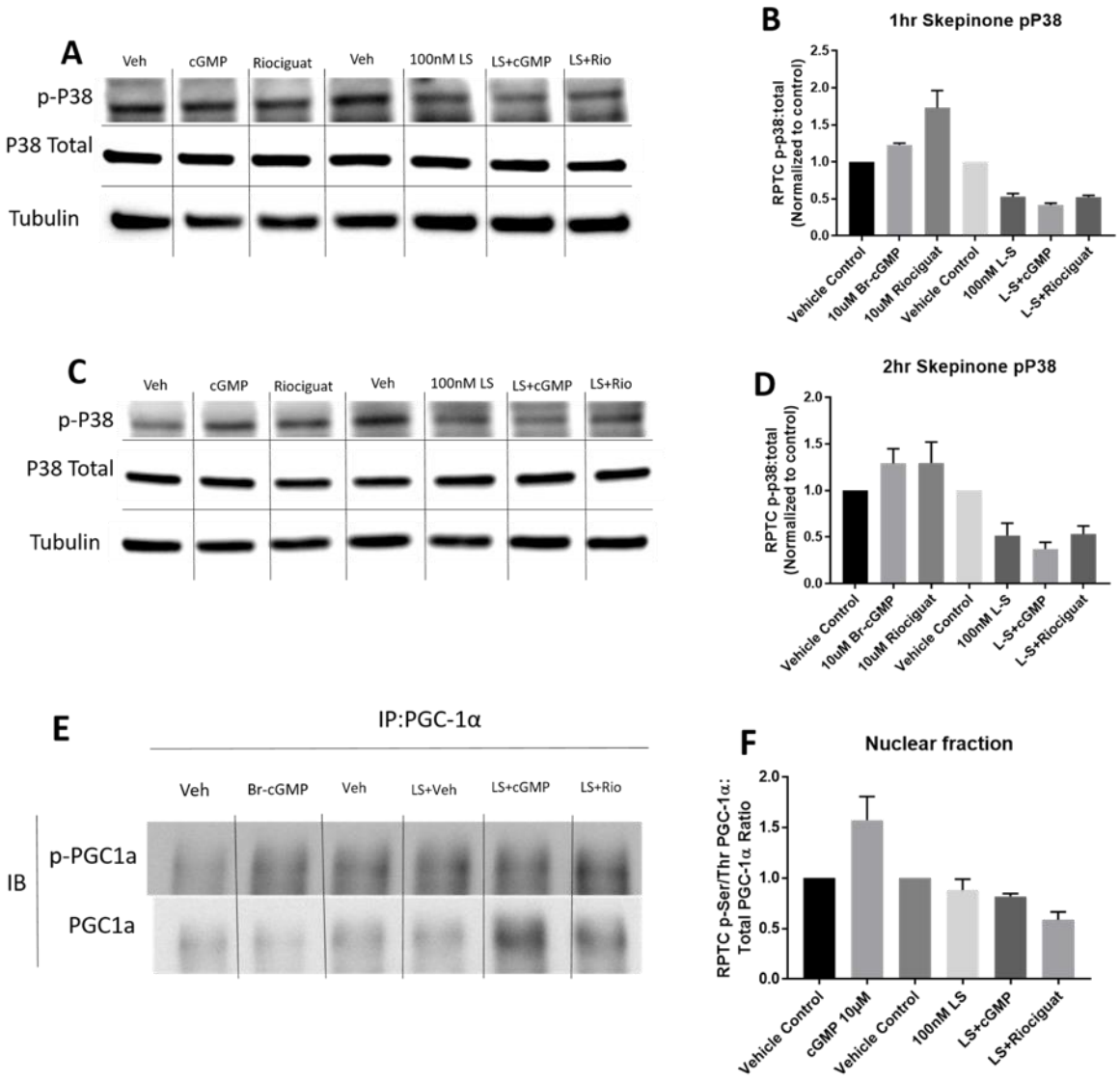

Figure 3.4: Inhibition of p38, inhibits PGC-1α phosphorylation at serine and threonine sites A and C) Representative blot for p-p38, p38 total, and tubulin after treatment with 30 min treatment with DMSO or 100nM L-Skepinone followed by exposure to DMSO, 8-Br-cGMP, and riociguat for 1 h and 2h. B and D) Densitometry analysis of p-p38 after 1h and 2h treatment respectively. E) Representative blot of phosphorylated serine and threonine residues following immunoprecipitation of PGC-1α by immunoblot analysis after 30 min treatment with DMSO or 100nM L-Skepinone followed by exposure to DMSO, 8-Br-cGMP, and riociguat in nuclear fractions. Total PGC-1α expression was measured after immunoprecipitation. F) Densitometry analysis for phosphorylated serine and threonine residue in the nuclear fractions. Data are represented as mean S.E., N=7-8 for A-D, N=4-5 for E-F. * represents significance compared to Vehicle ($p<0.05$).

PGC-1α mRNA increases at 4 h with 8-Br-cGMP

Phosphorylation of PGC-1α at Threonine 298, Threonine 262, and Serine 265 by p38 can increase the half-life of PGC-1α and therefore increase transcription of PGC-1α as well[62]. We have previously shown an increase in PGC-1α transcription at 24 h with 8-Br-cGMP however, the mechanism behind this increase is unknown[73]. Based on our results, we found an increase in the phosphorylation of PGC-1α at 1 h with 8-Br-cGMP. We wanted to see if there would be a rise in PGC-1α mRNA after this phosphorylation event. We measured PGC-1α mRNA in RPTC dosed with vehicle, 10μM 8-Br-cGMP, and 10μM riociguat. We found a 1.5-fold increase in PGC-1α mRNA (Figure 3.5).

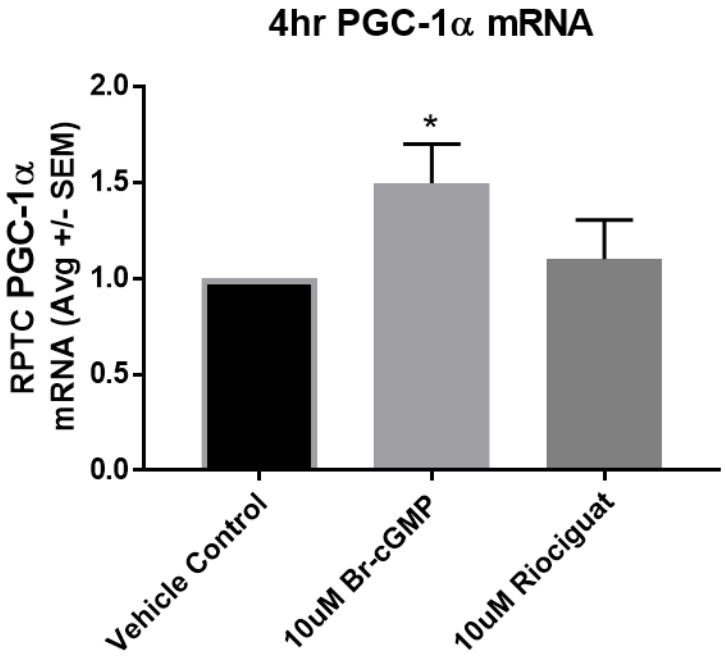

Figure 3.5: PGC-1a mRNA increases at 4 h with 8-Br-cGMP PGC-1α mRNA was measured after RTPC were dosed with DMSO, 10uM 8-Br-cGMP, and10uM Riociguat for 4 h. Data are represented as mean S.E., N=5 * represents significance compared to Vehicle ($p<0.05$).

Discussion

Previous studies from our lab have shown that cGMP can induce MB in RPTC[73]. We dosed with 10μM 8-Br-cGMP for 24 h and found an increase in PGC-1α mRNA as well as the mRNA for other mitochondrial proteins[73]. However, the mechanisms for how cGMP can induce MB is unknown and still under investigation for different cell types. Here we have elucidated the pathway between cGMP and PGC-1α in renal proximal tubule cells (RPTC).

We treated RPTC for 1 h with DMSO, 10μM 8-Br-cGMP and 10μM riociguat, an sGC stimulator that targets the reduced/heme-dependent form. The reduced form predominates in naïve cells and therefore, we included riociguat in all of our experiments for comparison. We measured PKG activation by measuring the phosphorylation of VASP (pVASP) at serine 239 and we measured PGC-1α protein in Figure 3.1. The increase in pVASP proved that our dosing of 8-Br-cGMP was effective since PKG is a direct target of cGMP. We expected to see no change in PGC-1α protein or mRNA (data not shown) since the dosing was only for an 1 h and this time point is too short. The half-life of PGC-1α is approximately 31 min and therefore phosphorylation of PGC-1α is a possible event that may occur within an hour of dosing[334]. Phosphorylation of PGC-1α by p38 at specific sites such as Threonine 298, Threonine 262, Serine 265 increases the stability of PGC-1α[335]. In Figure 3.2, we show that PGC-1α is phosphorylated at serine and threonine residues and is located in the nucleus when RPTC are dosed with 8-Br-cGMP. Another important finding in Figure 3.2, is that KT5823, the PKG inhibitor, inhibits phosphorylation of PGC-1α. We hypothesized that activated p38 phosphorylates PGC-1α

at Threonine 298, Threonine 262, Serine 265 in the cytosol then translocates to the nucleus to sustain PGC-1α transcription.

p38 is a downstream-associated target of PKG. Several studies have shown a correlation between both proteins[329]. Isolated cardiomyocytes dosed with sodium nitroprusside (SNP), an NO donor, activated p38 through a cGMP-dependent mechanism[336]. Lipopolysaccharide (LPS)-stimulated human neutrophils produced NO and cGMP leading to the activation of p38 through PKG[337]. Although other studies may suggest that p38 regulation by NO can be cGMP-independent, here we show that p38 phosphorylation is downstream of PKG and its activation is inhibited by KT5823 in Figure 3.3. Moreover, p38 inhibition also inhibited phosphorylation of PGC-1α at serine and threonine residues as we showed in Figure 3.4.

The effect of riociguat on cGMP signaling in RPTC is not clear. We found a slight increase in pVASP, although not significant, in the presence of riociguat. Regulation of sGC activity or expression of its subunits in naïve RPTC are unknown and therefore it is possible that longer exposure to riociguat is required to see a robust effect of riociguat on this pathway. An hour treatment may have proved to be insufficient. Targeting sGC is upstream of cGMP production and therefore may prove to be more difficult to elucidate signaling since the amount of cGMP produced in the presence of riociguat may not be equivalent to the effect of 10μM 8-Br-cGMP.

Here we propose a pathway between cGMP and PGC-1α in RPTC mediated by PKG and p38. Further investigation is required to validate this pathway for other cell types.

Chapter 4: Conclusions and Future Directions to the Role of Soluble Guanylyl Cyclase Signaling in Mitochondrial Biogenesis and Renal Injury

Conclusions:

Here we have presented a study discussing the role of sGC in regulating mitochondrial biogenesis (MB) in renal proximal tubule cells (RPTC) and in renal injury. We proposed opposing roles of sGC. In chapter two, we proposed that sGC plays a role in suppressing MB in the early phase of I/R. On the other hand, in chapter three, we elucidated a signaling pathway from cGMP production to PGC-1α transcription and translation in RPTC.

In chapter two, we showed that sGC activity is slightly increased after I/R. However, the redox state of sGC is altered after injury. We utilized the pharmacological differences between riociguat and cinaciguat to target two different redox states of sGC. We discovered that after I/R, the reduced form of sGC decreases. Interestingly, renal cortical tissue cGMP also decreased and this finding is consistent with the decreased presence of the reduced state of sGC. If the heme moiety in sGC is oxidized cGMP production will be compromised. However, PKG was activated 24 h after I/R suggesting that cGMP production after I/R was sufficient enough, even though these levels are decreased at the tissue level. One plausible explanation of this increase in sGC activity after I/R, can be found at 3 h after I/R. At 3 h after I/R, Akt phosphorylation is increased. Endothelial NOS is a target of Akt if phosphorylated at serine 1177 and is increased at 3 h after I/R[338]. The reactive oxygen species introduced with I/R injury as well as the increased production of NO can increase sGC activity after I/R.

After discovering that PKG is activated I/R, we explored potential downstream targets of PKG such as ERK1/2 and p38, both of which were increased after I/R. Phosphorylation of FOXO1, the downstream target of ERK1/2, decreased at 24 h after I/R suggesting that ERK1/2 is not regulating FOXO1 at this time point. This data also showed that the resulting FOXO1 signaling is independent of PKG.

It is not certain, whether ERK1/2 or p38 is the mediator between PKG activation and decreased MB. To explore the role of protein in this process, we utilized trametinib, a MEK1/2 inhibitor that successfully inhibits the phosphorylation of ERK1/2. Surprisingly, we discovered that pretreatment with trametinib attenuated the phosphorylation of eNOS at 3 h after I/R. Moreover, PKG activation seemed to be trending upwards at 3 h after I/R and trametinib had no effect. However, knowing that ERK1/2 inhibition attenuates renal injury, this ERK1/2 induced PKG activation may have a renoprotective effect. Although this piece of data may hint at ERK1/2 involvement in sGC suppression of MB, further investigation is necessary.

In chapter three, in our *in vitro* studies we show that 8-Br-cGMP, a cGMP analog, activates PKG. We verified this activation by measuring the phosphorylation of VASP, vasodilator stimulated phosphoprotein. Moreover, we show that there is also an increase in p38 MAPK activation. If PKG activation is inhibited, p38 activation is also inhibited. This finding shows that p38 is a downstream target of PKG, although is not directly phosphorylated by PKG. Further studies are needed to elucidate this PKG/p38 connection. Interestingly, p38 can directly phosphorylate PGC-1α on Threonine 262,

Threonine 298, and Serine 265 therefore increasing the half-life of PGC-1α and increasing its transcription[62]. We show an increase in the phosphorylation of PGC-1α at serine and threonine sites in the presence of 8-Br-cGMP. Surprisingly, inhibiting PKG also inhibited the phosphorylation of PGC-1α. Finally, to prove that p38 is the mediator between PKG and the phosphorylation of PGC-1α, we used a p38 inhibitor, L-Skepinone. Pre-treatment with L-Skepinone attenuated the increase in the phosphorylation status of PGC-1α proving that p38 is indeed the mediator.

These findings are novel in that this particular mechanism exists in RPTC. These experiments included riociguat, an sGC stimulator. Although riociguat slightly increased phosphorylation of VASP and increased p38 activation at 1 h, it is not certain whether riociguat increased the phosphorylation of PGC-1α. It is possible that this event may not occur within an hour of exposure. Here in chapter two, we show a positive role for sGC in inducing MB in RPTC.

Remaining Questions

Although in both chapters we have provided proof for a role for sGC in regulating MB, there are several unanswered questions that should be addressed to better elucidate sGC signaling in RPTC and in renal injury.

In terms of renal injury, the first question regards the importance of sGC in renal injury. The function and activity of sGC is a complex process and depends on the

microenvironment of the enzyme. Different types of insults can trigger different signaling pathways that begin with the activation of sGC. To answer this question, apo-sGC mice can be useful in elucidating sGC signaling. Apo-sGC mice contain a point mutation in the beta 1 subunit, the catalytic subunit of sGC, where the heme moiety is attached to the amino acid, phenylalanine instead of histidine[250]. This allows for improper ligation of heme moiety. As discussed in detail in chapter two, sGC activity in apo-sGC mice is decreased in the presence of nitric oxide donors[250]. It would be worthwhile to measure PGC-1α mRNA and protein as well as mitochondrial DNA copy number. Other mitochondrial proteins should be measured as well. If sGC plays a vital role in regulating MB, our hypothesis is that MB would be decreased in these mice. Secondly, to determine if sGC is absolutely necessary in the recovery or prevention of renal injury, apo-sGC mice should undergo I/R. It is possible that these mice may not even survive the surgery since cGMP production is dysfunctional and cGMP is necessary for vasodilation. However, if the apo-sGC mice do survive the surgery, markers of MB should be measured.

The second unanswered question revolves around sGC function and activity. Post-translational modifications of sGC include S-nitrosation and phosphorylation although, phosphorylation is an event that occurs rarely and is still under investigation. S-nitrosation of sGC can occur under conditions of oxidative and nitrosative stress. In conditions of I/R, S-nitrosation of sGC can decrease cGMP production[245,339]. Measuring S-nitrosation of sGC, although difficult, would give more insight into the microenvironment of sGC after I/R.

sGC function can depend on the presence of other proteins such as Hsp90[340]. Hsp90 is a chaperone heat shock protein that is associated with sGC[341,342]. Hsp90 can assist with inserting the heme moiety in the catalytic subunit of sGC[343]. Successful heme insertion allows for proper ligation and stabilizes the sGC heterodimer[344]. Hsp90 dissociates from the mature dimer allowing for NO to bind[340]. In conditions of oxidative stress or when the heme is oxidized, Hsp90 will re-associate with the beta 1 subunit as shown in Figure 4.1. It would be interesting to investigate the role of Hsp90 after I/R and if it is bound to sGC. Based on our results thus far, we would hypothesize Hsp90 association to sGC since we detected a decrease in the reduced state of sGC after I/R. Exploring the role of Hsp90 would provide insight into sGC function in our model of I/R.

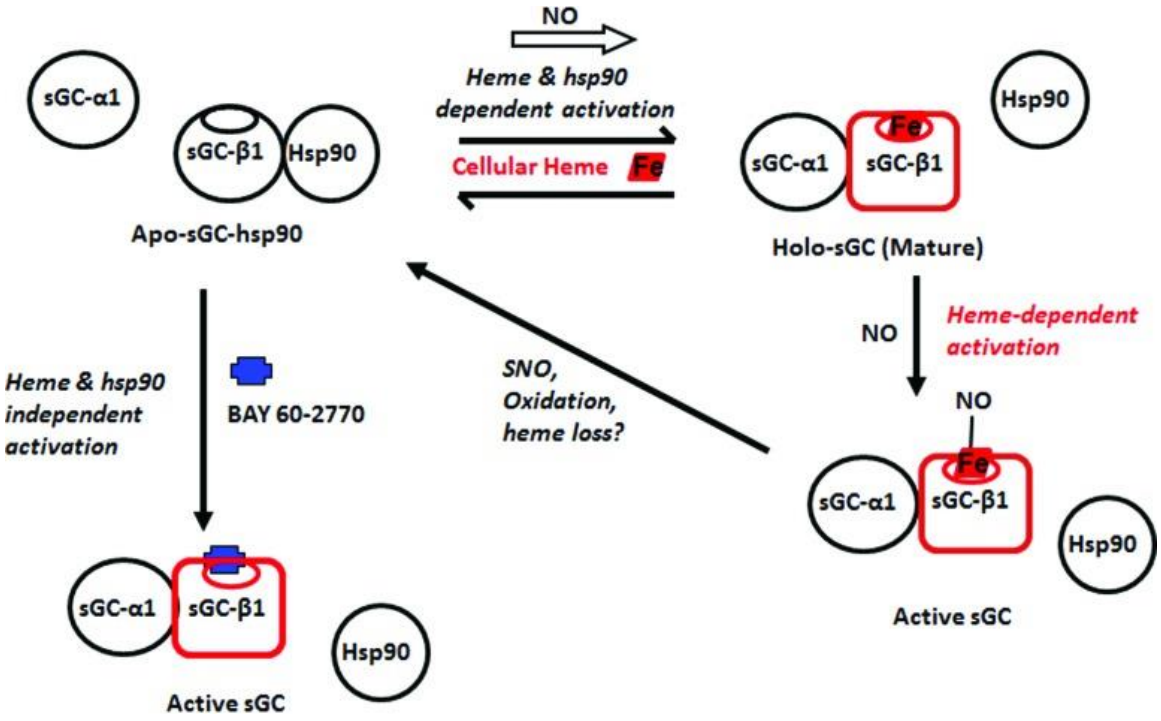

Figure 4.1: sGC NO response, maturation, and exclusive interaction of sGC-β1 with hsp90 versus sGC-α1. An equilibrium exists in cells between an hsp90-bound apo-sGC-β1 and a heme-replete sGC-β1 that is associated with sGC-α1. NO can rapidly shift this equilibrium to the right when cell heme levels are sufficient and hsp90 is active. NO can then bind to the heme in the sGC heterodimer and activate catalysis (lower right). Further NO exposure may cause S-nitrosation of sGC-β1 and heme oxidation/loss and thereby desensitize sGC toward NO and promote its hsp90 reassociation. Binding of the heme-independent activator, BAY 60-2770 (blue), to the apo-sGC-hsp90 species can occur independent of active hsp90 and cellular heme, and this triggers the same changes in sGC-β1 structure and protein interactions that are needed to activate its catalysis. From: Ghosh, A. & Stuehr, D. J. Regulation of sGC via hsp90, Cellular Heme, sGC Agonists, and NO: New Pathways and Clinical Perspectives. *Antioxid. Redox Signal.* **26**, 182-190, doi:10.1089/ars.2016.6690 (2017)

The final question that will be discussed here is the pathway between PKG and mitochondrial biogenesis. As mentioned above in chapter two, we provide evidence for possible ERK1/2 involvement. However, there is a possibility that other mediators can play a role in the pathway. First, we will address p38 as a potential mediator. To properly address this, we would administer a p38 inhibitor, SB203580, at two doses 10 mg/kg and 3 mg/kg 1 h before I/R. It is not certain whether the increase in the phosphorylation of p38 is part of activating the inflammatory response therefore possibly playing a detrimental role or if it is necessary for the recovery from I/R. At either dose, SB203580 should inhibit the phosphorylation observed 24 h after I/R. If p38 plays a role in regulating MB, then we hypothesize that either dose of SB203580 should attenuate renal injury and prevent the decrease in MB. It is well known that p38 can regulate the inflammatory response by triggering the transcription of pro-inflammatory cytokines such as IL-6, TNF-a, and IL-8[345-347]. It is possible that inhibiting p38 may primarily inhibit this inflammatory response and have no effect on MB.

It is not clear how PKG may be involved in the phosphorylation of p38. Several studies have shown that PKG can directly phosphorylate MEKK1 in its N-terminal domain[290]. MEKK1 is an upstream target of MAPK proteins such as JNK and p38[348]. Although it's mostly associated with the JNK pathway, crosstalk exists between both the JNK and p38 pathways (Figure 4.2)[349]. MEKK1 can directly activate an upstream targets of p38[349,350]. It would be worthwhile to measure MEKK1 24 h after I/R, as well in the *in vitro* studies we discussed in chapter three. In the case of our *in vitro* studies, although we showed

inhibition of p38 by inhibiting PKG activation, measuring MEKK1 activation in these experiments would better elucidate our proposed pathway.

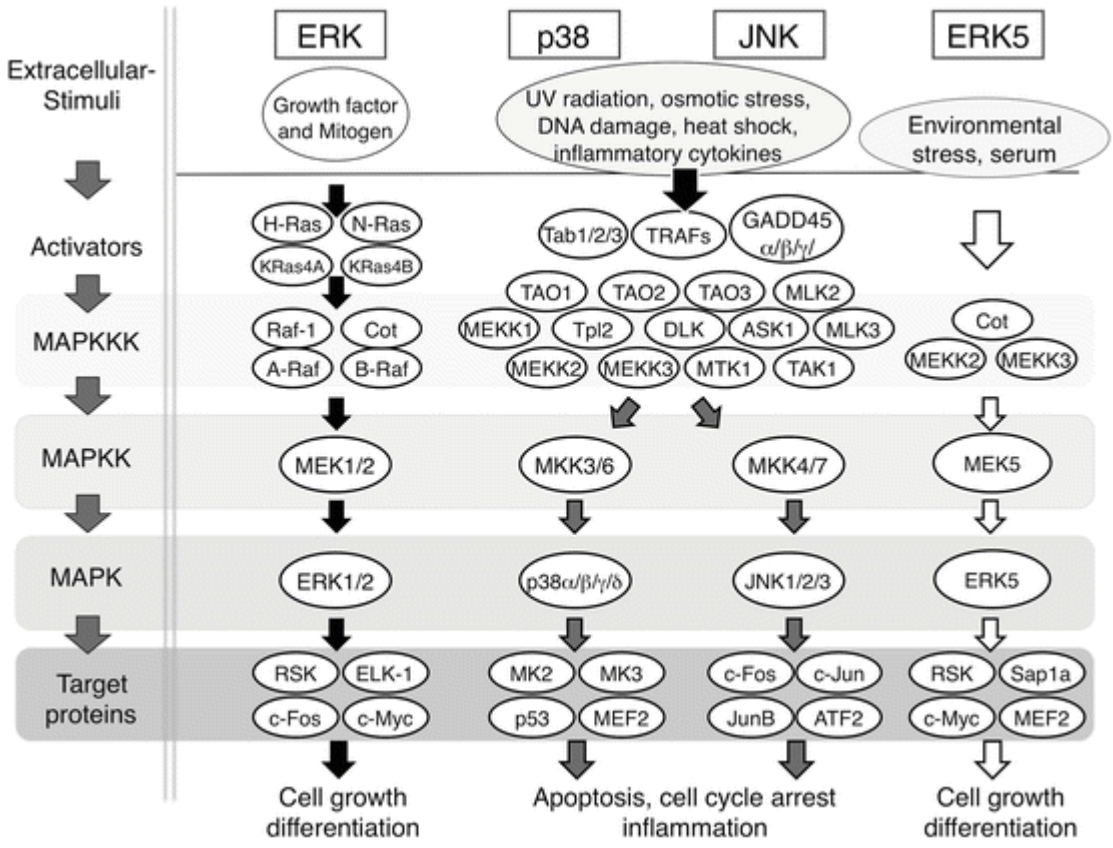

Figure 4.2: Overview of mammalian mitogen-activated protein kinase (MAPK) signaling cascades From: Takekawa, M. & Kubota, Y. in *Protein Modifications in Pathogenic Dysregulation of Signaling* (eds Jun-ichiro Inoue & Mutsuhiro Takekawa) 211-231 (Springer Japan, 2015)

Although elucidating the role of MEKK1 may provide better insight especially in our *in vitro* studies, it does not address how phosphorylated p38 or ERK1/2 contribute to decreased MB at 24 h in our *in vivo* studies. Therefore, we thought about targets of PKG that play a role in maintaining mitochondria function. In the early phase of I/R, several groups have observed mitochondrial swelling and fragmentation and cell death[169,351-354]. There is an increase in calcium levels inside the mitochondria and ATP levels are decreased[355,356]. These events trigger the opening of the mitochondrial permeability transition pore (MPTP)[356]. In recent years, the role of a mitochondrial potassium channel (mitoK$_{ATP}$) that is located in the inner membrane, has gained attention in cardiac I/R. Ischemic preconditioning in the heart can open mitoK$_{ATP}$, releasing ROS from the mitochondria and reducing damage. Opening of these channels has shown to prevent the opening of MPTP, thereby providing a cardioprotective role for mitoK$_{ATP}$[357,358]. PKG can target mitoK$_{ATP}$ leading to a release in ROS and subsequent activation of ERK1/2 (Figure 4.3)[268,359,360]. The interaction between PKG and mitoK$_{ATP}$ is still under investigation. Some groups suggest that PKG transmits this cardioprotective signal to PKCe that is bound to the mitochondrial inner membrane[361,362]. PKCε is activated and directly phosphorylates mitoK$_{ATP}$ causing it to open[361,363]. This pathway was demonstrated in cardiomyocytes however, it is still unknown if this protective effect exists in renal I/R.

Our previous studies regarding ERK1/2 in renal I/R prove that inhibiting ERK1/2 is renoprotective[313,314]. The mechanism described above in the case of cardiac I/R shows that phosphorylation of ERK is important to elicit cardioprotective effects. It seems that the importance of phosphorylated ERK1/2 is contradictory in terms of PKG-dependent

effects. Therefore, it would be beneficial to explore the role of PKG-dependent activation of mitoK_{ATP} and whether this compensatory mechanism is activated during I/R and if ERK1/2 does help elicit downstream effects. Taken together, the pathway from PKG to decreased MB needs further investigation as simultaneous events up until 24 h after I/R may contribute to sustained suppression of MB.

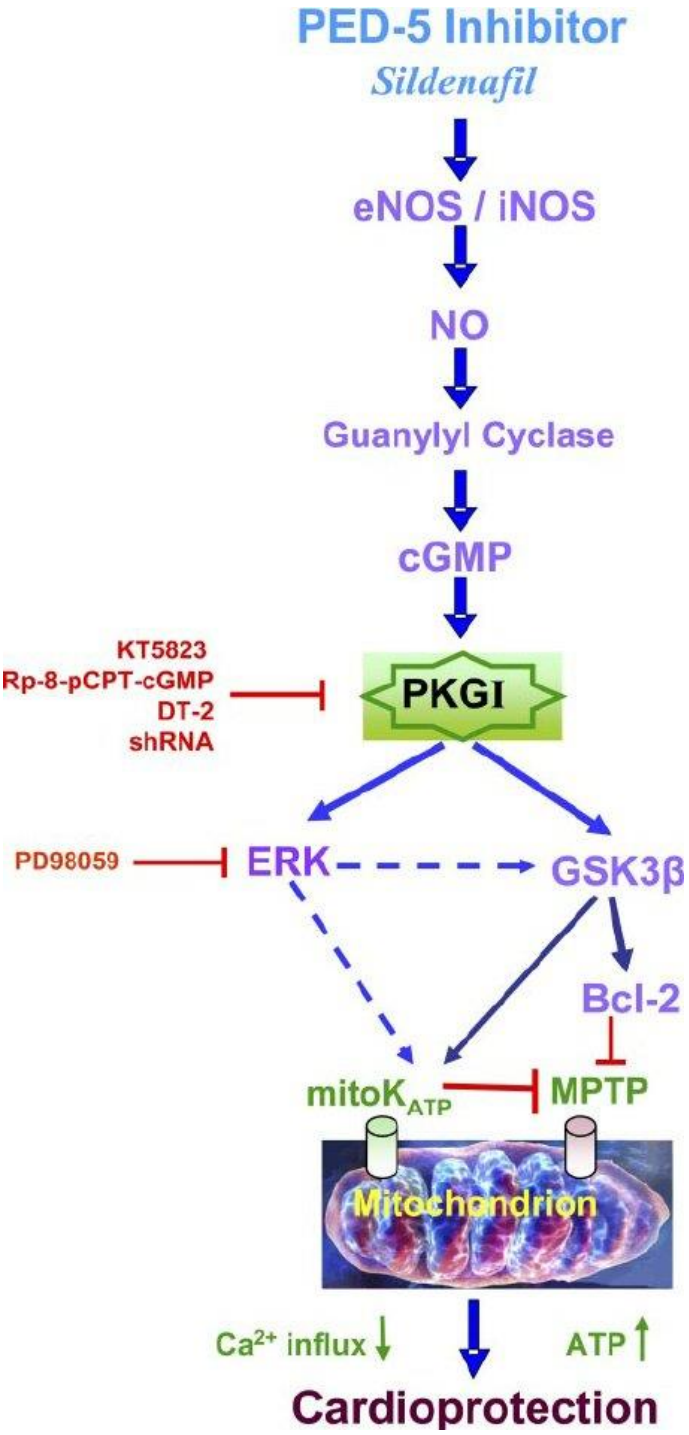

Figure 4.3: Proposed signaling pathways of PKG-dependent cardioprotection by sildenafil against myocardial ischemia-reperfusion injury. From: Das, A., Xi, L. & Kukreja, R. C. Protein kinase G-dependent cardioprotective mechanism of phosphodiesterase-5 inhibition involves phosphorylation of ERK and GSK3beta. *J. Biol. Chem.* **283**, 29572-29585, doi:10.1074/jbc.M801547200 (2008).

Appendix: M2 Phenotype Predominates in the Recovery Phase, 144 h after Ischemia-Reperfusion-Induced Acute Kidney Injury.

Introduction

In renal ischemia and reperfusion injury (I/R), events that take place from the early phase of I/R to the late phase are intricate and complex. Within the first 24 h after I/R, there is an influx of reactive oxygen species (ROS) and a significant increase in the release of proinflammatory damage-assocated molecular patterns (DAMPs), hypoxia-inducible factors (HIFs) and adhesion molecules from the injured tubular epithelium[364,365]. Pro-inflammatory cytokines and the release of these molecules facilitate the recruitment of many types of immune cells such as neutrophils, monocytes, dendritic cells, B cells, T cells, and NK cells[365]. A few studies have shown that preventing the influx of certain immune cells such as neutrophils can reduce the amount of renal damage and accelerates recovery[366].

Resident immune cells in the kidney such as macrophages increase in number after I/R[367,368]. The microenvironment created by proinflammatory cytokines, DAMPS, and HIFs activate resident macrophages as well as infiltrating monocytes to adopt a proinflammatory phenotype[367]. These proinflammatory macrophages produce NO, adding to the ROS already present and inflict further tubular injury. Studies have beeen conducted showing that although macrophage depletion in the early phase of injury may be beneficial, macrophage depletion in the recovery phase decreases renal repair[369-373]. These studies imply that macrophages have a wide variety of characteristics that adapt to the microenvironment from the early phase of I/R to the late recovery phase. Macrophages can be defined in terms of a M1/M2 phenotype, where M1 macrophages are proinflammatory and M2 macrophages exhibit anti-inflammatory properties as shown

in Figure A1.1. The characteristics defining the M1/M2 phenotype are not absolute, rather they are complex and can be overlapping.

	M1 phenotype	M2 phenotype
Use of Arginine	Oxidize L-Arginine to Citrulline and nitric oxide (inducible NOS)[374]	Hydrolyze Arginine to Ornithine and Urea (Arginase 1)
Cytokine production	Pro-inflammatory: TNF-α, IL-6, IL-23, IL-12[375]	Anti-inflammatory: IL-10, IL-1ra, TGF-β
Transcription factors	STAT1, IRF5[376,377]	STAT6, IRF4, KLF-4[378]
Macrophage Marker expression	CD86, CD80, IL-1R, TLR4	CD163, CD206 (Mannose Receptor), Msr1 (Macrophage Scavenger Receptor 1)

Figure A1.1: Comparison between M1 and M2 macrophage phenotype

Here we attempted to characterize the M1/M2 phenotype in our model of I/R. The M1 phenotype generally releases iNOS, or inducible NOS, and are activated by IFN-y and/or LPS[367]. They secrete proinflammatory cytokines such IL-6, IL-12, and TNF-a. M2 macrophages, alternatively, express mannose receptor (MR), arginase 1 (Arg1), and macrophage scavenger receptor-1 (Msr1)[367,379]. The driving force behind the M1 or M2 phenotype is macrophage colony-stimulating factor (M-CSF/Csf1) and granulocyte macrophage colony-stimulating factor (GM-CSF/Csf2). It seems the both are necessary for macrophages to adopt an M2 phenotype[380,381]. Inhibition of Csf2 before I/R, suppresses tubular repair while Csf1 can inhibit tubular apoptosis and facilitate tubular repair[381,382].

In this study we measured Csf1, Csf2, MR, Msr1 and Arg1 in our model of I/R. We focused on the early phase of injury, 24 h after I/R, and the recovery phase, 144 h after I/R.

Material and Methods

Ischemia-reperfusion (I/R)-induced AKI mouse model

Mice were subjected to bilateral ischemia where both renal pedicles were clamped for 18 min at 37°C and then removed to reintroduce blood flow as previously described.[155] Sham mice were operated on in the same manner except for no clamping. Mice were sacrificed either 24 h or 144 h after injury depending on the experiment and blood was collected via retro-orbital bleed. All tissues were flash frozen immediately.

For LY344864 administration, mice were injected with 2 mg/kg intraperitoneally once daily, starting 24 h after I/R. Mice were sacrificed 144 h after I/R. All tissues were flash frozen immediately.

Serum Creatinine Measurement

Blood was collected via retro-orbital bleed. Blood was spun down at 10,000xg at 10 min. The supernatant was collected and immediately stored in the -80C. Serum creatinine was measured using the Diazyme kits per manufacturer's protocol.

qRT-PCR

RNA was isolated from renal cortical tissue using Trizol (Life Technologies). cDNA was produced using the iSCRIPT Advanced cDNA Synthesis Kit (Biorad) according to the manufacturer's protocol. SsoAdvanced Universal SYBR Green Supermix reagent (BioRad) was used with the generated cDNA according to manufacturer's protocol.

mRNA expression was determined using a 2-triangle triangle CT method where mouse actin RNA was used for normalization.

Results

Csf1, Csf2, Msr1, MR, and Arg1 mRNA expression is increased 144 h after I/R

We measured mRNA expression of all markers at 24 h after I/R. We found a significant increase in Csf1 mRNA expression in the renal cortex at 24 h (Figure A1.2a). However mRNA expression of the other markers such as Csf2, Msr1, and MR did not increase at 24 h after I/R (Figure A1.2b-e). Arginase 1 mRNA expression decreased after I/R although this comparison to sham mice was not significant (Figure A1.2d).

In the midsection (total) of the kidney, we found no change in the mRNA expression of Csf2, Msr1, Arg1, and MR 24 h after I/R (Figure A1.2b-e). However, we found a significant increase in Csf1 mRNA expression 24 h after I/R (Figure A1.2a).

We measured all five markers at 144 h after I/R and found a significant increase in the mRNA expression in the renal cortex and in the total kidney (Figure A1.2).

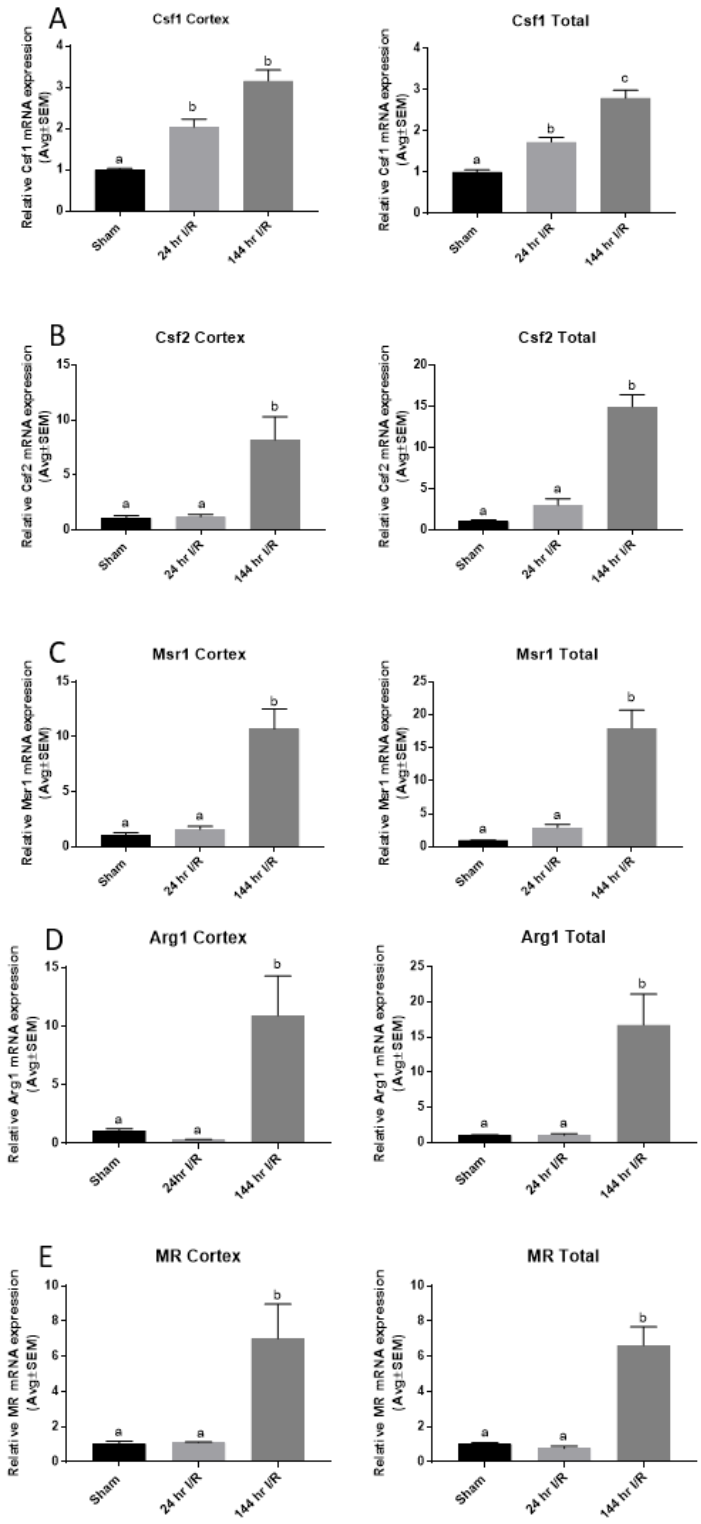

Figure A1.2: Csf1, Csf2, Msr1, MR, and Arg1 mRNA expression is increased 144 h after I/R mRNA expression of Csf1, Csf2, Msr1, MR, and Arg1 subunits following 24 and 144 h I/R. Data are represented as mean S.E., N=6-11. b represents significance compared to Sham, c represents significance compared to both sham and 24 h after I/R ($p<0.05$)

Csf1 mRNA expression positively correlated with delta serum Creatinine and serum creatinine at 144 h after I/R

We sought to investigate if a correlation existed between Csf1 mRNA expression and serum creatinine after I/R. We found two correlations significant for Csf1 mRNA expression. First, we measured serum creatinine at 144 h after I/R injury in all mice, including sham mice. We found a positive correlation in that as serum creatinine at this time point was attenuated near sham levels, Csf1 mRNA expression increased in both the renal cortex and in the total kidney shown in Figure A1.3a. Secondly, we measured delta serum creatinine for each animal subtracting serum creatinine measured at 144 h from serum creatinine measured at 24 h after I/R. The increase in delta serum creatinine indicates recovery from I/R. We found that as delta serum creatinine increased, so did Csf1 mRNA expression in both the renal cortex and in the total kidney as shown in Figure A1.3b.

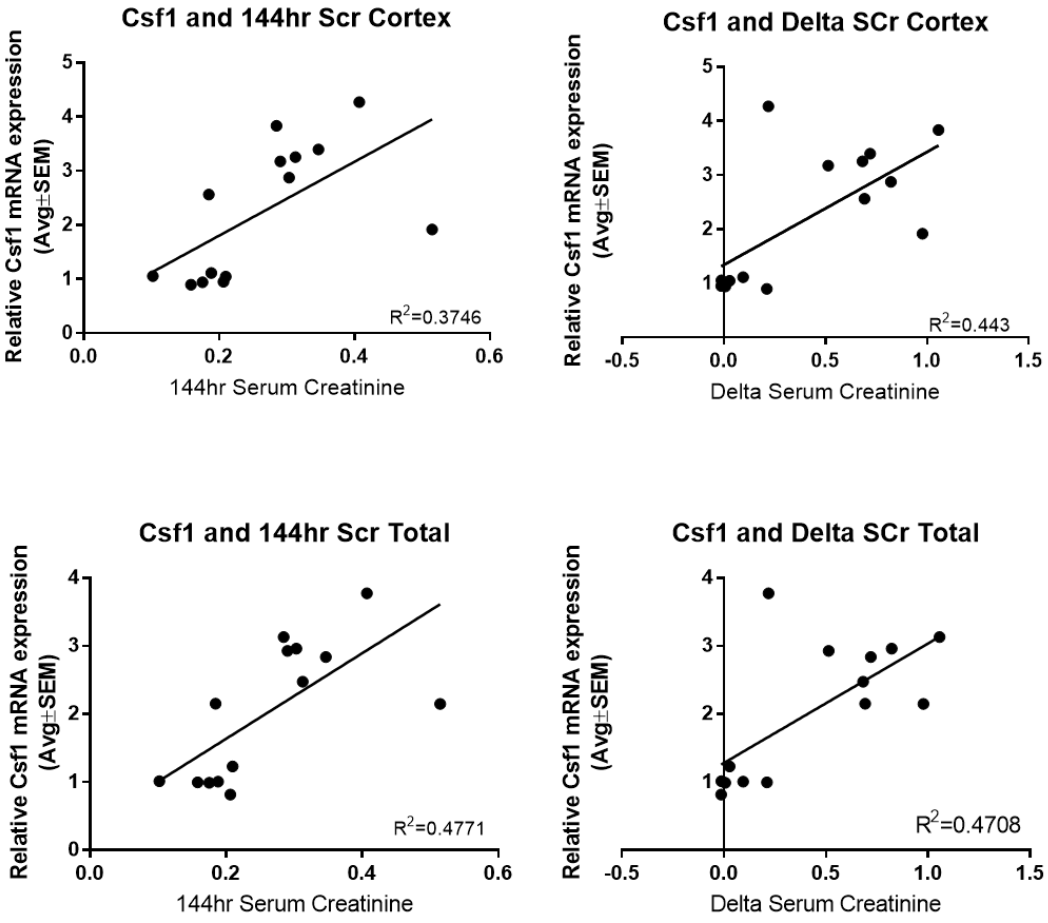

Figure A1.3: Csf1 mRNA expression positively correlated with delta serum Creatinine and serum creatinine at 144 h after I/R mRNA expression of Csf1 at 144 h I/R in the renal cortex and total kidney correlated with serum creatinine at 144hr and delta serum creatinine. Significance is represented with a R^2 value.

As delta serum creatinine increased, so the did the mRNA expression for Arg1, MR, Msr1, and Csf2

Similar to Figure A1.4, we made two correlations between serum creatinine and mRNA expression of all markers as shown in Figure A1.3. As serum creatinine at 144 h after injury decreased, mRNA expression for MR, Msr1, and Csf2 increased. Secondly, as delta serum creatinine increased, mRNA expression for all markers also increased.

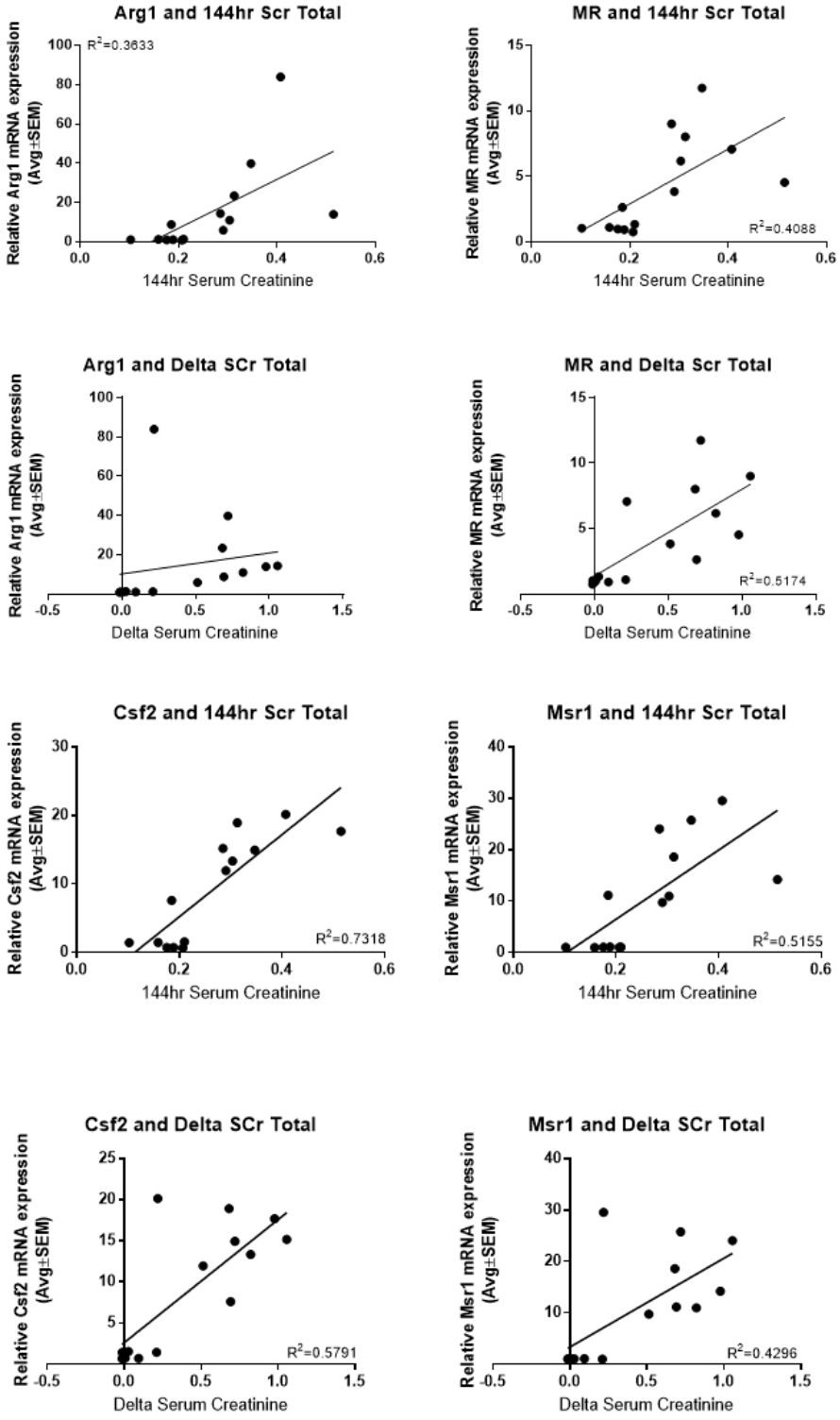

Figure A1.4: As delta serum creatinine increased, so the did the mRNA expression for Arg1, MR, Msr1, and Csf2 mRNA expression of Arg1, MR, Msr1, and Csf2 at 144 h I/R in the renal cortex and total kidney correlated with serum creatinine at 144hr and delta serum creatinine. Significance is represented with a R^2 value.

LY344864 decreases Arginase 1 mRNA expression 144 h after I/R

Previously in our lab, we have shown accelerated renal recovery in our model of I/R, after administration of LY344864, once daily starting at 24 h after I/R, for 144 h. We also showed recovery of mitochondrial biogenesis. Therefore, we were curious to see the effect of LY344864, a 5-HT_1F agonist, on the mRNA expression of these markers. LY344864 had no effect on the mRNA expression of all of the markers except arginase 1. We found a significant decrease in arginase 1 after treatment with LY344864 at 144 h after I/R in the renal cortex, but not in the total kidney (Figure A1.5).

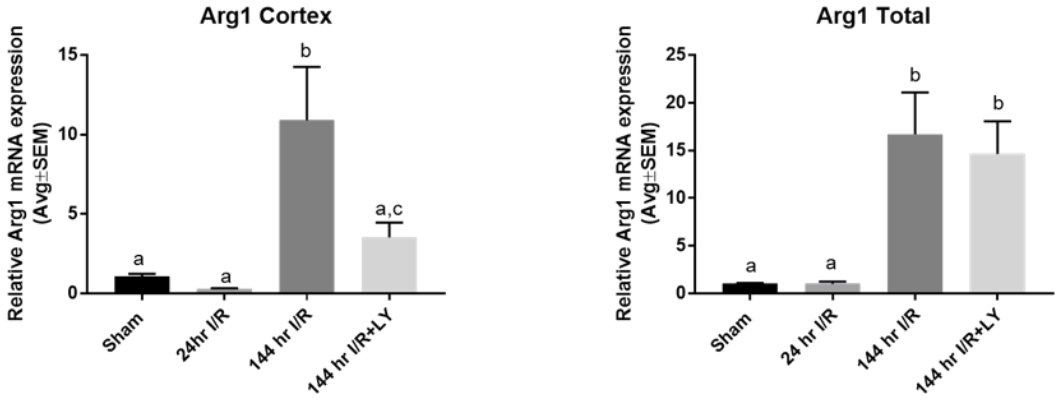

Figure A1.5: LY344864 decreases Arginase 1 mRNA expression 144 h after I/R mRNA expression of Arg1 at 24 and 144 h I/R in the renal cortex and total kidney. mRNA expression of Arg1 at 144 h with LY344864 treatment. Data are represented as mean S.E., N=6-11. b represents significance compared to Sham, c represents significance compared to both sham, 24 h after I/R, and 144 h after I/R ($p<0.05$)

Discussion

In this study, we wanted to characterize the M1/M2 phenotype in our model of I/R. We focused on the early phase of injury, which is 24 h after I/R and is also the peak of maximal renal dysfunction. We also focused on the recovery phase of I/R, which in our model occurs at 144 h after I/R.

We found a significant increase in all markers at 144 h after injury in the renal cortex and in the total kidney (Figure A1.2). These increases are consistent with other studies that have shown that expression of arginase 1 and mannose receptor by M2 macrophages predominate in the recovery phase and their expression should be decreased in the early phase of injury. We compared the serum creatinine at 144 h as well as the delta serum creatinine with the mRNA expression of all these markers (Figure A1.3 and A1.4). Our results are consistent with other studies in that the recovery of each animal, as determine by increased delta serum creatinine, is associated with an increase in all five of these markers.

Interestingly, Csf1 expression is increased in the renal cortex at 24 h after I/R although not significant. At 144 h after I/R, Csf1 mRNA expression is further increased in the renal cortex and in the total kidney. Csf1 is a cytokine that mediates crosstalk between macrophages and tubular cells[383,384]. Although further investigation is needed to elucidate this signaling, our data support theories regarding positive crosstalk between macrophages and tubular cells for renal repair[384]. The role of Csf2 is still unclear however, it is known that its expression is necessary for renal repair after I/R.

Previously in our lab, we have shown the attenuation of renal function and recovery of mitochondrial biogenesis, MB, with the treatment of LY344864, a 5-HT_{1F} agonist[180]. In Figure A1.5, we were curious to see the effect of LY344864 on our markers. Except for Arginase 1, LY344864 did not have any effect on our other markers (data not shown). The decrease in arginase 1 mRNA expression due to LY344864 treatment is surprising. In cardiac I/R the prolonged presence of arginase 1 is not favorable for recovery[385]. Since we have shown previously that LY344864 attenuates renal function compared to sham mice, it is possible that one of the effects of LY344864 is decreasing arginase 1, providing a new insight into the importance of arginase 1 in the recovery phase of renal I/R. The mechanism behind LY344864 induced MB was elucidated in renal proximal tubule cells where the involvement of endothelial NOS was important[386]. A balance exists between eNOS expression and arginase 1 expression where both enzymes use L-arginine to function. Moving forward, it would be worthwhile to investigate this balance between eNOS and arginase 1 expression in the presence of LY344864 144 h after I/R.

Here, we have provided consistent evidence for the M2 phenotype 144 h after I/R in the recovery phase and have introduced an interesting effect of LY344864, that still needs to be elucidated.

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