

SJMLS-1-2016-24

Interaction of Hypoxia inducible factor 1 α (HIF 1- α) and Peroxisome proliferator-activated receptor α (PPAR- α) in the protection against ischemia reperfusion injury in the rat kidney.

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Abstract

In response to reduced oxygen availability, cellular and molecular adaptive, cellular and molecular adaptive changes occur in living organisms. Interaction of hypoxia-inducible factor (HIF) and peroxisome proliferator-activated receptor (PPAR α) has been identified as an important mechanisms of cellular adaptation to low oxygen. This study tested the hypothesis that inhibition of prolyl hydroxylase domain (PHD) through activation of PPAR α confers protection against ischemia/reperfusion injury. In animals subjected to ischemia with or without PHD inhibitor L-mimosine (L-MM) (600mg/kg). Renal Blood Flow (RBF) recovered to 93 \pm 8%, 56 \pm 7% (p < 0.001) and 87 \pm 10% (p < 0.001) in control, ischemic and L-mm treated groups respectively after 48hr of reperfusion. Coincident with these changes, serum nitric oxide decreased by 33 \pm 3% in the ischemic group while L-MM treatment abolished the effect of ischemia (p <0.05). 24hr urine volume increased by 100 \pm 00% (p <0.001) and 3 \pm 0% (p >0.05) in ischemic and L-MM treated groups respectively. Similarly, urinary protein excretion (24hr) increased 5-fold (p <0.001) in the ischemic group but was attenuated to a 2-fold increase by L-MM (p < 0.05). However, when mice knock out of PPAR- α (-/-) were subjected to the same treatment, L-MM failed to exhibit any significant effect in any of the studied analytes. These findings suggest that stabilization of HIF-1 α via inhibition of PHD through PPAR- α activation protects against Ischemia reperfusion injury (IRI) in the rat kidney.

Keywords: HIF-1 α , PPAR α , PHD and Ischemic kidney.

Introduction

Renal ischemia is a major cause of acute renal failure (ARF) which is faced in many medical and surgical conditions such as shock, kidney transplantation, partial nephrectomy, renal artery angioplasty and in many urological operations (Bhalodia *et al.*, 2009). In these conditions, abrupt restoration of blood flow after the ischemia reintroduced oxygen within cells that damages cellular proteins, DNA and the plasma membrane. Damage to cell membrane may in turn cause the release of more free radicals species, leukocytes build up in small capillaries, obstructing them and leading to more ischemia (Wayne, 2005). This initiates a complex and interrelated sequence of events resulting in injury to and the eventual death of renal cells (Paller, 1998). Several factors have been implicated in the pathophysiological changes occurring during renal I/R. These include vascular or micro vascular injury, endothelial dysfunction, accelerated cell necrosis, granulocytes activation, oxidative stress and modulation of nitric oxide/angiotension II axis (Adams and Raji, 2000).

The kidney is particularly prone to ischemic injury (Hill *et al.*, 2008). Though it was reported that blood flow to the kidney is high, the presence of oxygen (O₂) shunt diffusion between arterial and venous vessels keeps renal tissue oxygen tensions relatively low (Nangaku *et al.*, 2008). The kidney is very sensitive to changes in oxygen delivery as it is required for various energy demanding activity such as basolateral activity of Na/K-ATPase and for basic

reabsorptive, filtering and secretory functions of the kidney (Nangaku et al, 2008).

Hypoxia-inducible factor 1 (α), is a transcription factor that was recognized as a master switch of cellular adaptation to reduced oxygen availability and many hypoxia-triggered protective mechanisms are hypoxia-inducible factor (HIF)-dependent. More than 100 target genes have been identified to be modulated by HIF 1 α in the protection of cells against hypoxic insult (Wanja et al., 2011). Many of these genes are expressed to increase the capacity of cells or tissues to adapt when oxygen supply is reduced. These include genes encoding erythropoietin (EPO), which stimulates red cell production, and vascular endothelial growth factor, which stimulates angiogenesis, both serving to increase O₂ delivery; and glucose transporters and glycolytic enzymes, which facilitate ATP production in the absence of O₂. Physiologically however, HIF 1 α stabilization, is attenuated by the activity of an enzyme, prolylhydroxylase domain (PHD) which hydroxylates specific prolyl residues on HIF 1 α for subsequent rapid degradation of HIF1 α by proteasomes (Nangaku et al., 2008). HIF-1 α stability through PHD inhibition therefore, appears as a promising therapeutic target for the prevention of cells against ischemic injuries. Additionally, peroxisome proliferator-activated receptor alpha (PPAR- α) a group of lipid-activated nuclear receptors that heterodimerize with the 9-cis retinoic acid receptors (RXR) to form functional transcription factors that regulate genes involved in lipid metabolism, where reported to increase glucose metabolism and reduce fatty acid oxidation in hypoxic environment (Kodde et al., 2006). PPAR- α has a significant influence on mitochondrial respiration. It induces the expression of pyruvate dehydrogenase kinase (PDK), which inhibits the enzyme pyruvate dehydrogenase by phosphorylation. Thus, pyruvate is not converted into acetyl-CoA, preventing pyruvate entry into the TCA cycle (Sugden et al., 2002). As consequence, the mitochondrial oxygen-consumption is downregulated and hypoxic ROS generation is attenuated.

It is therefore rational, that stabilization of HIF -1 α factor by PHD inhibition is through activation of PPAR- α which synergistically confer adequate protection against I/R injury. As participation of products controlled by HIF-regulated genes is important in the adaptation of the renal cells to hypoxic challenges (Zou and Cowley, 2003), PPAR- α activation may optimize glucose and oxygen utilization in hypoxia which generate sufficient

amount of ATP anaerobically without producing excessive amount of ROS if aerobic driven TCA cycle and mitochondrial respiration was followed (Weidemann and Johnson, 2008). These group of transcription factors that integrate resistance to oxidative stress, with the ability to detect and orchestrate the storage and metabolism of lipids, while sparing glucose (which can be burnt anaerobically), was inevitable in hypoxic conditions. PHD inhibition through PPAR- α activation therefore, can constitute novel strategies and be an effective measure in activating angiogenesis, tissue repair and protection against ischemic insults (Shen et al., 2009). To help elucidate these inter connecting pathways, we hypothesized that inhibition of prolylhydroxylase enzyme through activation of PPAR- α attenuate I/R-induced renal injury in the rat.

Materials and Methods

Unless specified otherwise in the text, all chemicals used were obtained from Sigma-Aldrich (St. Louis, MO, USA and are of analytical grade.

Animals: Adult male Sprague-Dawley rats (450-500g) obtained from Harlan Sprague-Dawley (Houston, Texas, USA) were used for the study. The animals were maintained in a room with lighting that was adjusted to produce a normal day-night cycle. They were maintained on a standard diet of Purina chow and allowed ad libitum access to water and food at least 3 days to become acclimatized to the housing condition before use in the experiments. All protocols were approved by the Institutional Animal Care Facility Committee, Texas Southern University, Houston. Rat were randomly divided into three groups. These includes sham operated controls, ischemia/reperfusion group and L-mimosine treated ischemia/reperfusion group.

Induction of Ischemia/reperfusion: Rats were anaesthetized with ketamine (100mg/kg,i.p.) and left lateral flank incision was made. The left kidney was exposed and then, (1) the renal artery and vein were identified and occluded for 30 minutes with non traumatic artery clamp (Chatterjee and Thiemermann, 2003). Ischemia was confirmed visually by blanching of the kidney which signified successful occlusion. (2) At the end of the occlusion period, the clamp was removed and reperfusion allowed for 3 hours. During the reperfusion period, animals were placed on a thermostatically controlled warming table to maintain the animal's body temperature at 37°C. The incision site covered with gauze soaked with normal saline to prevent

evaporation. At the end of the 3 hours of reperfusion, kidneys were subjected to scanning (Moor laser Doppler imager, LDI V5.0; Moor Instruments Limited; Axminster, Devon, UK.) to measure the renal blood flow. Briefly, the laser beam was scanned over the kidney surface. Moving blood in the microvasculature caused a Doppler frequency broadening of the scattered laser light. The captured light were photo detected in the imager and then electronically and digitally processed to build up a color image display of the blood flow. After the scanning, the incision was closed, muscular layer with chromic gut 4.0 absorbable suture, and the skin layer with silk 4.0. In Sham-operated controls, the rats were treated identically except that the kidneys were only exposed but not clamped. A group of animals was treated with L-mimosine, 600mg/kg dissolved in 10% NaHCO₃ adjusted to Ph 7.4 which was administered to rats by oral gavage, 6 hours before induction of ischemia.

In an attempt to show that HIF 1 α stabilization by PHD inhibition is through PPAR- α , four groups of mice (n=4) in each, comprising of PPAR α receptor knock out (-/-) and wild type (+/+) were subjected to I/R after treatment with L-mm (600mg/kg) and vehicle in both the genotypes. Kidney scan was also taken and all the animals were returned to metabolic cages for 24 hours urine collection. Scanning was repeated after 24 hours of reperfusion in all the animals and after 48 hours in the rats only. At the end of the experiment, all the animals were anesthetized with pentobarbital (50mg/kg) and kidney tissues were harvested, snap frozen in liquid nitrogen and stored at -86^oC. Blood sample was collected through cardiac puncture for biochemical analysis. Measurement of Biochemical parameters. Serum Creatinine was measured by Jaffe Method (Tilkian et al, 1979), and serum level of nitric oxide by Griess method (Tsikas D 2005), Urinary volume was measured by gravimetry and urinary protein was determined by Bio-Rad method (Le Bricon, 2002).

Statistical analysis.

Changes in perfusion unit and other biochemical analytes were expressed as different from basal values. Data were analyzed with Prism statistical software and presented as mean \pm S.E.M. Group

comparisons were made using one way ANOVA followed by Bonferroni post hoc test. In all cases $p \leq 0.05$ was considered as significant.

Results

In this study, we investigated the possible inhibition of prolylhydroxylase enzyme through activation of PPAR- α attenuate I/R-induced renal injury in the rat. RBF was measured in sham, ischemic (I/R+Vehicle) and in ischemic rats treated with L-mimosine (I/R+LMM). Reading was taken as perfusion percentage relative to our preocclusion value. * $p < 0.001$ in the L-mm group when compared to vehicle. Figure 1 shows the renal blood flow (RBF) in the rat kidney. NO was measured in sham, ischemic (I/R+Vehicle) and in ischemic rats treated with L-mm (I/R+LMM). L-mm abolished the decrease caused by I/R. * $p < 0.05$ in the L-mm group when compared with vehicle. Figure 2 show the serum nitric oxide (NO) concentration in the rat. Urine protein was measured in sham, ischemic (I/R+Vehicle) and in ischemic rats treated with L-mm (I/R+LMM). L-mm abolished the increase caused by I/R. * $p < 0.05$ in the L-mm group when compared with vehicle. Figure 3 shows the Urinary protein concentration (24h) in the rat. UV was measured in sham, ischemic (I/R+Vehicle) and in ischemic rats treated with L-mm (I/R+LMM). L-mm abolished the increase caused by I/R. * $p < 0.05$ in the L-mm group when compared with vehicle. Figure 4 shows the Urine volume (UV) excretion (24h) in the rat. Serum creatinine was measured in sham, ischemic (I/R+Vehicle) and in ischemic rats treated with L-mm (I/R+LMM). L-mm abolished the increase caused by I/R. * $p < 0.05$ in the L-mm group when compared with vehicle. Figure 5 shows the Serum creatinine concentration in the rat. RBF was measured in sham (WT and KO), ischemic (I/R-WT+Vehicle, I/R-KO+Vehicle) and in ischemic mice treated with L-mimosine (I/R-WT+LMM and I/R-KO+Vehicle). Reading was taken as perfusion percentage relative to our preocclusion value. ## $p < 0.001$ in the L-mm-WT group when compared to I/R-KO-LMM. ** $p < 0.05$ in the I/R-WT-Vehicle compared to I/R-KO-Vehicle at 24h. # $p < 0.05$ in the I/R-WT-LMM group compared to I/R-KO-LMM at 3h. Figure 6 shows the Renal blood flow (RBF) in the mice.

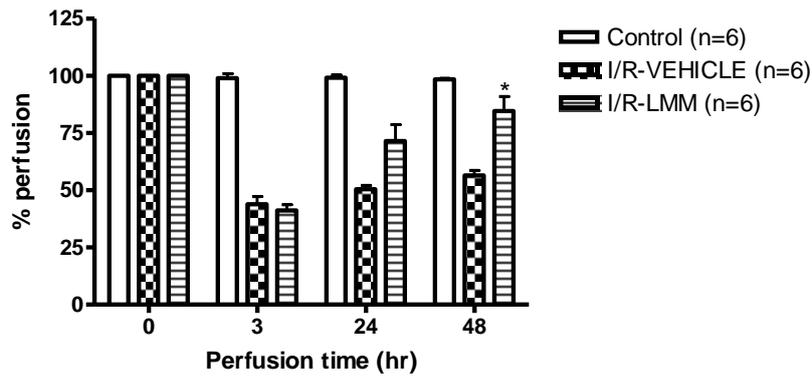


Figure 1. Renal blood flow (RBF) in the rat kidney.

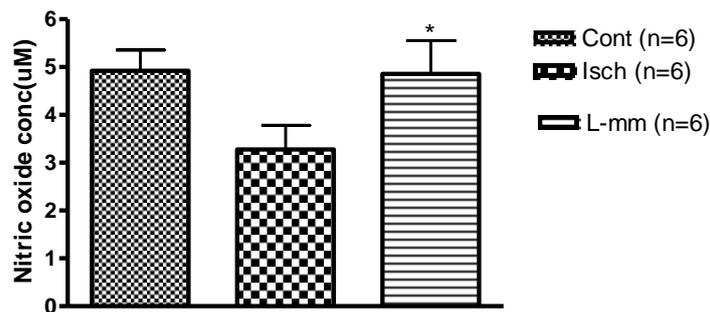


Figure 2. Serum nitric oxide (NO) concentration in the rat.

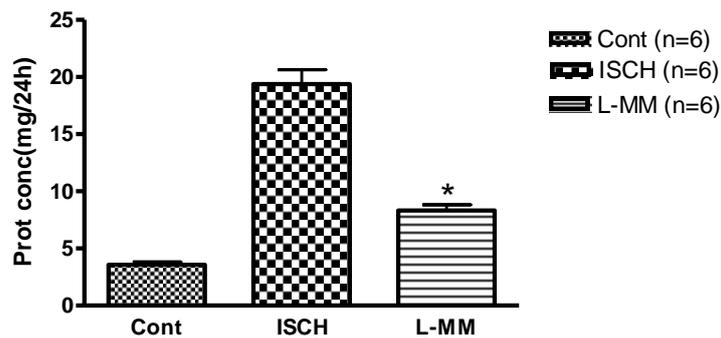


Figure 3. Urinary protein concentration (24h) in the rat.

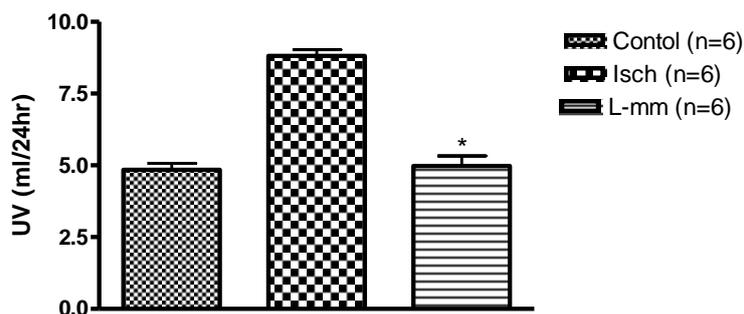


Figure 4. Urine volume (UV) excretion (24h) in the rat.

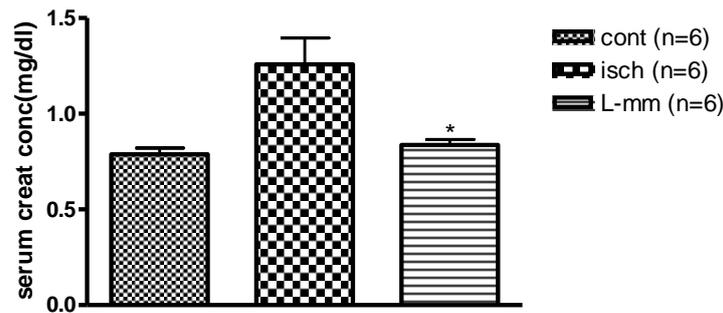


Figure 5. Serum creatinine concentration in the rat.

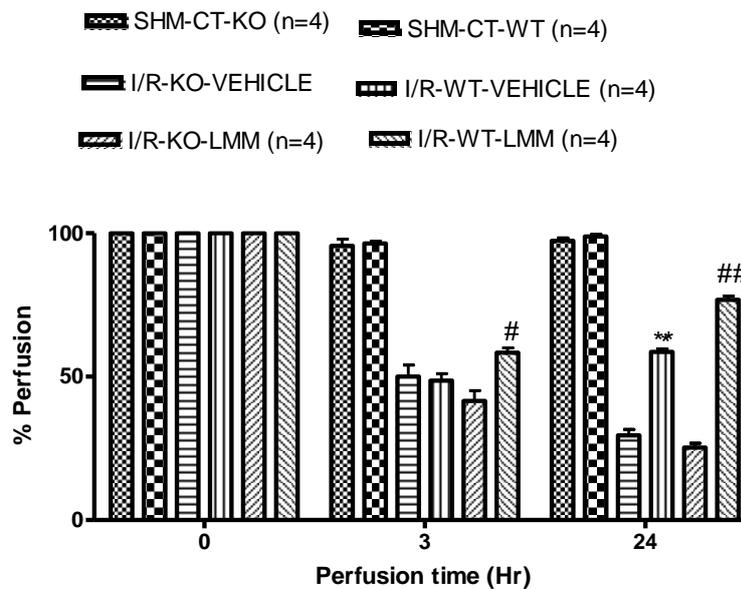


Figure 6. Renal blood flow (RBF) in the mice.

Discussion

Adequate supply of oxygen and other nutrients to the tissues of the body are required to maintain adequate cellular functions and the amount of blood flow to a particular organ is dramatically adjusted to meet metabolic demands. Ischemia occurs when the blood supply is inadequate to meet the tissue's metabolic demands an episode that is followed by a number of biochemical processes which include hypoxia, insufficiency of metabolic substrates and accumulation of metabolic waste.

Data from the current study showed that there was protection against ischemia / reperfusion following inhibition of prolyl hydroxylase domain (PHD) and activation of PPAR α as there are evidence that PPAR α is partially under the regulatory control of HIF 1 α or at very least by hypoxia itself (Nangaku, 2009). This observation points to the existence of a regulatory link between pathways controlled by the two transcription factors HIF 1 α and PPAR α . Thirty minutes of ischemia followed by 48 hours of reperfusion does not restore renal blood flow to pre-ischemic level in our I/R model, but treatment with L-mimosine (600 mg/kg) restored renal blood flow

to near pre-ischemic level. There is less levels of blood to the medullary than the cortical capillaries in the post-ischemic kidney, which can arise from leukocyte activation, enhanced leukocyte-endothelial interactions, which can promote injury and swelling of the endothelial cell, physically thereby causing obstruction to blood flow. In other ways, leukocytes can contribute to the production of local factors promoting vasoconstriction, and add to the effects of vasoconstriction on local blood flow and tubule metabolism (Shereidan and Bonventre, 2000). One can hypothesize that increased blood flow in the reperfusion may result in tissue damage due to rapid reoxygenation, changes in pH or intracellular ion transport (Bojakowski et al, 2001). Christol et al. (1993) reported about 30% reduction in the RBF in the rat's kidney, and the value remains significantly smaller than the pre-ischemic levels. The effect is attributed to swelling or contracture, which compressed capillaries, capillary endothelial cells damage with swelling preventing flow and obstruction of small vessels by leukocytes which adhere to the injured endothelial cells.

Nitric oxide (NO) is an important mediator of biological processes such as vascular homeostasis, neurotransmission, immunity and inflammation. These protective roles appears to have been compromised in our ischemic model as reported above. While some studies stated that NO potentiates the cytotoxic effects of I/R (Wang and Zweir, 1996). Previous report (Hercule and Oyekan, 2000; Webb et al., 2004; Duransky et al., 2005 and Newaz et al., 2006) have shown that NO plays important protective role in many disease conditions. During ischemia, e NOS activity is compromised as a results of its essential dependence on oxygen and the increasing acidosis (Graldez et al., 1997). After I/R, injured vascular cells and adherent leukocytes produce free radicals and inactivate NO, which may promote vasoconstriction and therefore the reduced perfusion (Heemann et al., 2000). The beneficial actions of nitric oxide are reported to be seen only in the absence of pathology. With I/R injury, the production of cytokines that inactivate NOS and activates iNOS. iNOS generates toxic levels of NO which react with abundant superoxide generated from I/R injury to produce the notorious vasoconstriction.

HIF-1 α has been shown to activate gene transcription of many sensitive genes such as nitric oxide synthase (Jung et al., 2000). NO also regulates neutrophil recruitment by inhibiting the expression of adhesion molecules in the vascular endothelium (Lascalzo and Welch, 1995). In this current study,

I/R of the kidneys appears to mediate decrease in the activity of e NOS with attendant decrease in NO synthesis. In the current studies, I/R mediates increased urinary protein excretion. Renal vasculature and proximal tubular injury is also a feature in response to I/R. In contrast to proximal tubules, the renal vasculature lacks efficient regenerative capacity leading to a persistent 30-50% reduction in vascular density following I/R (Basile et al., 2001). Reduction in vascular density may have important functional consequences following hypoxia by impairing haemodynamic and sodium regulatory responses. The impairment in vascular responses occurs in the presence of hypoxia. In this study, I/R apparently predisposed the renal cells to injury with apparent loss of glomerular filtration and tubular reabsorption and concentrating ability resulting in proteinuria and increased urine volume. As HIF-1 α transcriptionally regulates the expression of several enzymes such as NOS, COX-2 and HO-1 (Komatsu and Hadjiargyrou, 2004), which produce antihypertensive factors in the kidneys. PPAR α agonists are associated with anti-inflammatory effects that can modulate the initial cascade of genes induced in response to inflammatory stimuli (Zhan et al., 2009). The up regulation of HIF-1 α by PHD inhibitors and activation of PPAR α may contribute to the protection of renal structure and function as evidenced by decreased urinary protein excretion in treated ischemic rats.

Evidence available so far suggests that the generation of oxygen radicals as a results of renal ischemia and reoxygenation is of crucial importance (Wanja et al., 2006). In addition to converting hypoxia, HIF-1 α dependent gene expression can provide resistance to oxidative stress (Siddiq, 2007). This is because many of the genes regulated by HIF-1 α (e.g., erythropoietin, VEGF, MnSOD) can by themselves prevent oxidative stress-induced death (Zelko and Folz, 2005). Moreover, since activation of HIF-1 α pathway is associated with a shift away from oxidative energy metabolism towards anaerobic energy metabolism, a metabolic switch also reported to be mediated by PPAR α PHD inhibition through PPAR α activation shows protection against I/R -induced tubular damage. These was evidenced by increased urine excretion in I/R, that was attenuated by pretreatment with L-mmm and fenofibrate. Ischemia coupled with subsequent reperfusion phase, may injure tubular cells by depleting intracellular ATP; disrupting intracellular calcium homeostasis; generating free radicals; and causing cellular changes that destroy the integrity of the tubular cells resulting into misguided filtration

(Bonventre and Weinberg, 2003). Decreased blood flow to the kidney in the post ischemic period can also damage the glomeruli and alter its homeostasis (Loutzenhiser et al., 2006), translating to the loss of glomerular function with subsequent polyuria

Acknowledgement

Authors want to acknowledge the Management of Usmanu Danfodiyo University, Sokoto-Nigeria for the study leave, Nigeria's Tertiary Education Trust Fund for sponsorship and Centre for Cardiovascular Diseases, Texas Southern University, Houston Texas USA for the opportunity to carry out the bench work.

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