Glomerular Hyperfiltration and Hypertension in Diabetes

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Glomerular Hyperfiltration and Hypertension in Diabetes

by

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy
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Abstract

In the present study, we investigated the pathophysiological mechanisms of the hemodynamic alteration in diabetes. Glomerular hyperfiltration occurs in the early stage of diabetes mellitus and has been recognized to promote the pathogenesis of diabetic nephropathy. We determine the role of TGF response and the vascular tone of Af-Art in diabetic hyperfiltration and we found that 1) tubular high glucose directly activates NOS1 and increases NO generation in the macula densa, which inhibits TGF response and increases GFR; 2) high glucose dilates renal Af-Art through GLUT1 and mediated by NOS3-derived NO generation; 3) in diabetes, blunted TGF mediated by NOS1 in the macula densa promotes glomerular hyperfiltration. The prevalence of hypertension is much higher in diabetic subjects than non-diabetic population. We studied the potential mechanisms of blood pressure regulation in diabetes, including TGF response and renal afferent arteriolar response to vasoconstrictors, and we found that 1) inadequate NOS1 in the macula densa enhances TGF, which restricts glomerular hyperfiltration and induces hypertension in diabetes; 2) hemodynamic responses to ANG II is increased in diabetes, which is associated with increased expression and activity of AT1 receptors in the Af-Art; 3) Ang II upregulates the expression and activity of Nox2 and Nox4 in the macula densa, which enhances TGF response.
Chapter One: Introduction

Diabetes mellitus

Diabetes mellitus is a major public health issue in the United States with a high and growing rate of morbidity and mortality. According to the National Diabetes Statistic Report 2014, 29.1 million or 9.3% of American have diabetes mellitus and it remains one of the leading causes of death in the United States. Metabolic disturbances, resulting from either the limitation in insulin production or the disruption of insulin sensitivity, lead to variety of complications all over the body including heart disease, stroke, diabetic retinopathy and diabetic nephropathy (Brownlee 2005).

Diabetic nephropathy

Diabetic nephropathy is characterized as a progressive kidney disease with the primary lesion in glomeruli. Diabetic nephropathy occurs in 20-30% patients with diabetes mellitus, which is the leading cause and responsible for more than 40% cases of end-stage renal disease (Molitch et al. 2004). While the pathophysiology of diabetic nephropathy has not been completely elucidated, it is recognized that the hemodynamic changes in microcirculation, in particularly, the glomerular hyperfiltration during the early stage of diabetes are involved with the glomerular damage and promotes the development of diabetic nephropathy in the later phases (Brenner et al. 1996, Helal et al. 2012, Ruggenenti et al. 2012).
**Diabetic hyperfiltration**

Elevations in glomerular filtration rate (GFR), also called glomerular hyperfiltration, have been observed in about 70% of type 1 (Bank 1991, Levine 2008) and 50% of type 2 diabetic patients (Keller *et al.* 1996, Levine 2008, Nelson *et al.* 1996, Vora *et al.* 1992), which have been associated with an increased risk for diabetic nephropathy and worse prognosis (Abdi *et al.* 2012, Levine 2008, Persson *et al.* 2010). Nevertheless, the pathogenesis of glomerular hyperfiltration in diabetes has not been fully understood and several mechanisms have been proposed, including primary vascular and primary tubular mechanisms. According to the vascular hypothesis, hyperfiltration results from imbalance between vasoconstrictive factors (Angiotensin II, thromboxane A2, endothelin-1 and its ETA receptor, and reactive oxygen species) and vasodilatory factors (NO, prostanoids, kallikrein-kinin, atrial natriuretic peptide, Ang 1-7 and hyperinsulinemia) (Anderson & Vora 1995, Bank 1991, Cherney *et al.* 2010a, Cherney *et al.* 2010b, Cherney & Sochett 2011, Levine 2008, Premaratne *et al.* 2005). The tubular hypothesis proposes that tubular growth and the sodium-glucose cotransporter-2 (SGLT2) enhance proximal tubular reabsorption, which reduces NaCl delivery to the macula densa and increases GFR via the tubuloglomerular feedback (TGF) mechanism (Nordquist *et al.* 2009, Pruijm *et al.* 2010, Thomson *et al.* 2001, Thorup *et al.* 2000, Vallon *et al.* 1997, Vervoort *et al.* 2005).

**Tubuloglomerular feedback in diabetic hyperfiltration**

The macula densa is a group of specialized epithelial cells located at the distal end of the thick ascending limb (TAL) of the kidney, serving as a sensor of luminal NaCl concentration. The TGF response describes a mechanism by which an increase in NaCl
delivery to the macula densa promotes the release and formation of ATP and/or adenosine, which then constricts the afferent arteriole (Af-Art) and induces a tonic inhibition of single nephron glomerular filtration rate (SNGFR) (Ollerstam et al. 1997, Ren et al. 2000b, Welch et al. 2000). The TGF response is modulated by various factors including Ang II (Ren et al. 2002b, Welch & Wilcox 1990), arachidonic acid metabolites (Kurtz et al. 1986, Welch & Wilcox 1990), atrial natriuretic factor (Huang & Cogan 1987), succinate receptor GPR91 (Peti-Peterdi et al. 2016, Toma et al. 2008, Vargas et al. 2009), connexins (Saez et al. 2003, Takenaka et al. 2008, Takenaka et al. 2011), superoxide (Liu et al. 2004b, Ren et al. 2002a, Welch et al. 2000) and NO (Ito et al. 1993, Liu et al. 2004b, Schnermann 1998, Welch & Wilcox 1997). The TGF response has been extensively studied by measurement of stop flow pressure or proximal-distal differences in SNGFR in db/db mice or STZ-induced diabetic rats. In both type 1 and type 2 diabetic animals, the TGF response was found to be inhibited or reset, which permits the GFR to increase (Thomson et al. 2012, Vallon et al. 1999, Vallon et al. 2002).

**Macula densa NOS1 in diabetic hyperfiltration**

NOS1 is the predominant NOS isoform expressed in the macula densa cells (Mundel et al. 1992, Wilcox et al. 1992) and NO generated by the macula densa blunts the TGF response (Liu et al. 2004a). Alternative 5’-end splicing of NOS1 mRNA results in at least three different N-terminal NOS1 protein variants, called α, β and γ (Brenman et al. 1996, Eliasson et al. 1997). Recently, several studies from our laboratory demonstrated the significance of TGF responsiveness mediated by the macula densa NOS1 in the long-term control of sodium excretion and blood pressure. We found that
NOS1β is the primary splice variant and contributes to most of the NO generation by the macula densa (Lu et al. 2010, Lu et al. 2016). Mice with deletion of NOS1 specifically from the macula densa exhibit enhanced TGF responsiveness and develop salt-sensitive hypertension following a high salt diet (Lu et al. 2016). However, the significance of the macula densa NOS1 splice variants in diabetes has not been investigated. It has been well established that enhanced NO production is associated with renal hemodynamic changes during the early stages of diabetes (Komers et al. 1994, Komers et al. 2000a, Mattar et al. 1996, Tolins et al. 1993). Selective NOS1 inhibition induces a stronger renal hemodynamic response including reduction of GFR in the diabetic compared with control animals (Komers et al. 2000a, Komers et al. 2000b, Levine et al. 2006, Thomson et al. 2004a). In addition, GFR did not further decrease by adding non-selection NOS inhibition in the presence of a NOS1 inhibitor (Thomson et al. 2004a). These observations demonstrated that the significance of NOS1-derived NO in the pathogenesis of glomerular hyperfiltration in the early stages of diabetes.

**Glucose, SGLT2 and SGLT1 in diabetic hyperfiltration**

High blood glucose is the hallmark and most significant metabolic disturbance of diabetes mellitus (Giugliano et al. 2008). Intravenous infusion of glucose increases GFR in both normal people and diabetic patients (Brochner-Mortensen 1973a, Brochner-Mortensen 1973b, Christiansen et al. 1981), as well as in experimental animals (Noonan et al. 2001, Woods et al. 1987). In addition, clinical studies have also demonstrated the significant role of blood glucose in hyperfiltration: glomerular hyperfiltration in diabetes improves after effective insulin therapy, but worsens with poor plasma glucose control.
(Magee et al. 2009). More than 99% of filtered glucose is reabsorbed in the proximal tubule by SGLT1 and SGLT2, two types of sodium glucose linked transporters. SGLT2 is present in the S1 and S2 segments of proximal tubule and accounts for more than 97% of renal glucose reabsorption, whereas SGLT1 is present in the S2 and S3 segment and accounts for the remaining 2-3% in normoglycemic conditions (Vallon & Thomson 2017). Unlike SGLT2 which is highly proximal tubule specific, SGLT1 was detected in small intestine, liver, lung, heart (Chen et al. 2010, Song et al. 2016, Vrhovac et al. 2015), as well as in TAL and macula densa (Balen et al. 2008). In diabetes, SGLT2 expression level increases (Vallon et al. 2014, Vestri et al. 2001, Vidotti et al. 2008), but SGLT1 expression is not consistent in reported studies, varying from increase (Vidotti et al. 2008), no change (Vestri et al. 2001), or decrease (Vallon et al. 2013).

The significance of SGLT2 mechanism has been extensively studied in diabetic hyperfiltration. Increased proximal tubular glucose and sodium reabsorption by SGLT2 reduces NaCl delivery to the macula densa, which increases GFR via TGF inhibition (Thomson et al. 2004b, Vallon et al. 1999). A recent EMPA-REG OUTCOME trial showed that SGLT2 inhibition reduced hyperfiltration, lowered blood pressure and reduced cardiovascular and all-cause mortality in patients with type 2 diabetes (Zinman et al. 2015). However, the significance of renal SGLT1, in particularly, macula densa SGLT1 in diabetes has not been investigated.

**Renal afferent arterioles in diabetic hyperfiltration**

As the primary preglomerular resistant vessels, renal afferent arterioles (Af-Arts) play a critical role in control of glomerular hemodynamics. The glomerular hydrostatic
pressure, the driving force of glomerular filtration, is reciprocally correlated with the resistance of Af-Arts (Gnudi et al. 2007, Loutzenhisier et al. 2004, Rosivall & Peti-Peterdi 2006). Several elegant studies, with isolated and perfused rabbit Af-Arts have demonstrated that an increase in glucose concentration dilates the Af-Art, which involves the local accumulation of succinate, activation of G protein-coupled metabolic receptor GPR91 in endothelium, and nitric oxide (NO) generation (Toma et al. 2008).

**Diabetes and hypertension**

Diabetes mellitus (DM) and hypertension are two major health issues in the United States. The prevalence of DM was 9.3% as of 2012, and hypertension affects about one-third of the adult population (Ong et al. 2008). The prevalence of hypertension in diabetic subjects is approximately twice that of the non-diabetic population (Epstein & Sowers 1992, Klein et al. 1984). Diabetic patients with hypertension exhibit an increased risk for diabetic complications, such as coronary heart disease, stroke, peripheral vascular disease, retinopathy and nephropathy (Chobanian et al. 2003, Colosia et al. 2013, Estacio & Schrier 2007, Gillespie & Hurvitz 2013, Haffner et al. 1998, Khairallah et al. 2007). However, the mechanisms underlying the high prevalence of hypertension in diabetic patients are not fully elucidated.

**Renin-angiotensin system in diabetes**

The renin-angiotensin system (RAS) plays an essential role in regulating blood pressure and is one of the most important therapeutic targets for hypertension (Crowley et al. 2005, Hall 1991a, Hall 1991b). RAS blockers, such as angiotensin-converting
enzyme inhibitors and angiotensin receptor blockers, have been recognized as the first-line treatment with additional beneficial effects for hypertensive patients with diabetes, which suggests the involvement of RAS in the development of hypertension in diabetic subjects (Arauz-Pacheco et al. 2004, Kirpichnikov & Sowers 2002, Reboldi et al. 2009). Although plasma renin was suppressed (Christlieb et al. 1976, Fernandez-Cruz, Jr. et al. 1981, Price et al. 1999) or normal (Beretta-Piccoli et al. 1981, Luik et al. 2003) in diabetic patients, the responsiveness of blood pressure to angiotensin II (Ang II) was demonstrated to be exaggerated in normotensive and mildly hypertensive patients with Type 1 diabetes (Drury et al. 1984, Weidmann et al. 1979). However, the mechanism of the enhancement in blood pressure response to Ang II remains elusive. A previous study with kidney cross-transplantation between wild-type and Ang II type 1 (AT1) receptor-deficient mice examined the action of AT1 receptors in the kidney. They demonstrated the predominant role of the renal AT1 receptors in the development and maintenance of Ang II-dependent hypertension (Crowley et al. 2006). Renal Af-Arts are the major resistant vessels in the kidney and play a critical role in regulating renal hemodynamics and blood pressure (Dilley et al. 1984, Gothberg et al. 1979, Knox et al. 1974, Loutzenhiser et al. 2006). However, the role of renal Af-Arts in enhanced response of renal hemodynamics to Ang II remains elusive.

Hypothesis

General hypothesis

Diabetes mellitus is a major public health issue in the United States. Glomerular hyperfiltration occurs in the early stage of diabetes mellitus and has been recognized to
promote the pathogenesis of diabetic nephropathy. However, the mechanism responsible for the diabetic glomerular hyperfiltration is not fully elucidated. High blood glucose is the hallmark and most significant metabolic disturbance of diabetes mellitus. Intravenous infusion of glucose increases GFR in both normal people and diabetic patients, as well as in experimental animals. As the primary pre-glomerular resistant vessels, renal Af-Arts play a key role in control of glomerular hemodynamics. Therefore, we would like to determine the effect of high glucose on TGF response and renal afferent arteriole. On the other hand, the prevalence of hypertension in diabetic subjects is approximately twice that of the non-diabetic population. The presence of hypertension in diabetic patients further exacerbates their morbidity and mortality. However, the pathophysiological mechanisms for the development of hypertension in diabetes have not been completely elucidated. Thus, I would like to investigate whether there are derangement in the mechanisms of blood pressure regulation in diabetes, which might account for the high prevalence of hypertension in diabetes including TGF response and renal afferent arteriolar response to Ang II (Figure 1).

**Hypothesis I.** An increase of tubular glucose directly activates NOS1 and increases NO generation in the macula densa, which inhibits TGF response and increases GFR.

**Hypothesis II.** High glucose dilates renal Af-Art through GLUT1 and mediated by NOS3-derived NO generation.

**Hypothesis III.** Blunted TGF mediated by NOS1 in the macula densa promotes glomerular hyperfiltration in diabetes, whereas inadequate NOS1 in the macula densa
restricts glomerular hyperfiltration, induces hypertension and exacerbates diabetic kidney injury.

**Hypothesis IV.** Enhanced hemodynamic responses to ANG II in diabetes are associated with increased expression and activity of AT1 receptors in the Af-Art.

**Hypothesis V.** Ang II upregulates the expression and activity of Nox2 and Nox4 in the macula densa, which enhances TGF response.

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**Figure 1. Glomerular hyperfiltration and hypertension in diabetes.**

We would like to determine the effect of high glucose on TGF response and the vascular tone of Af-Art, the two critical mechanisms in regulation of afferent arteriolar resistance and investigate the potential mechanisms of blood pressure regulation in diabetes, including TGF response and renal afferent arteriolar response to vasoconstrictors.
Chapter Two: Glomerular Hyperfiltration in Diabetes

Project I: plasma glucose increases GFR by directly enhancing NOS1 activity in the macula densa and blunting tubuloglomerular feedback

Introduction

There are approximately 29.1 million people with diabetes mellitus (DM) in the United States. DM increases the risk for many serious disorders and is one of the leading causes of death in United States. Diabetic nephropathy is one of major complications of DM (Gross et al. 2005, Selby et al. 1990, Tervaert et al. 2010). The pathophysiologic mechanism of diabetic nephropathy is complex and has not been elucidated. Increased GFR is generally considered as one of the initial risk factors for the development of DM (Zatz et al. 1985).

One of the major mechanism to regulate GFR is TGF, which is an intranephron negative feedback in each juxtaglomerular apparatus (JGA) between tubule and afferent arteriole. Increase of luminal delivery of NaCl to the macula densa initiates a TGF response by constricting the afferent arteriole mediated by adenosine and/or ATP (Bell et al. 2003, Liu et al. 2002a, Nishiyama & Navar 2002, Persson et al. 2002, Ren et al. 2004, Sun et al. 2001). TGF response has been proposed to play an important role in the development of diabetic hyperfiltration. Volkers proposed SGLT-TGF model for the diabetic hyperfiltration. The expression and activity of sodium glucose cotransporters
(SGLT), in particularly the SGLT2, in the proximal tubules are increased in early DM (Rahmoune et al. 2005, Vallon et al. 1999). This enhanced SGLT activity lowers the luminal NaCl delivery to the macula densa, which blunts TGF response and contributes to the increased SNGFR (Cherney et al. 2014, Stanton 2014). Several studies from Volker’s lab have clearly demonstrated that TGF is attenuated in diabetes, which contributes to the glomerular hyperfiltration (Vallon et al. 1995, Vallon 2003). Hall et al. found that acute intravenous glucose infusion increased renal blood flow and GFR in anesthetized dogs. By using nonfiltering kidneys, they further indicated a possible role of TGF in acute hyperglycemia-induced hyperfiltration (Woods et al. 1987).

However, it has not been clear whether glucose itself has any direct effect on the macula densa and TGF response. Glucose is freely filtered through the glomerular capillaries and is completely reabsorbed by proximal tubule in normal physiological condition, so the glucose at the macula densa is negligible. However, the amount of filtered glucose might exceed the maximal capacity of reabsorption by proximal tubule, leading to the elevated glucose delivery to the macula densa under some conditions, especially in DM.

In present study, we hypothesized that glucose directly activates NOS1 and enhances NO generation in the macula densa, which inhibits TGF response and increases GFR. We examined the direct effect of luminal glucose on nitric oxide generation by the macula densa, TGF-mediated vasoconstriction and GFR in normal C57BL/6 mice and macula densa specific NOS1 knock out (MD-NOS1KO) mice.
Methods

Animals. C57BL/6 mice (male, 13-15 weeks old) and New Zealand white rabbits (male, 1.5 to 2.0 kg) were purchased from Harlan Laboratories. The MD-NOS1KO mice were generated by our lab, as described previously (Lu et al. 2016).

Measurement of GFR in conscious mice. GFR was measured by the clearance of plasma FITC-inulin with a single bolus injection in conscious mice. Mice were lightly anesthetized with isoflurane and injected with the FITC-inulin solution (3.74 µl/g body weight) through the penile vein. Blood (10 µl) was collected into heparinized capillary tubes through the tail vein at 3, 7, 10, 15, 35, 55, 75, and 90 minutes after inulin injection. The blood samples were centrifuged at 8000 rpm for 5 minutes at 4˚C. Plasma (1 µl) was collected from each sample. FITC-inulin concentration of the plasma was measured using a plate reader (Cytation3, BioTek, VT) with 485-nm excitation and 538-nm emission. GFR were calculated with GraphPad Prism 6 (GraphPad Software) (Qi & Breyer 2009).

Basal GFR was measured first in all mice. After 5 days, one group of mice were intravenously infused of 50 µl glucose solution (2 M in saline) together with FITC-inulin (3.74 µl/g body weight) through the penile vein for measurement of GFR. Mice infused same amount of 0.9% saline together with FITC-inulin were used as control.

Measurement of TGF in vivo with micropuncture. Similar method for micropuncture was used as we previously described (Fu et al. 2012, Fu et al. 2013). Briefly, mice were anesthetized with inactin (80 mg/ml, i.p.) and ketamine (50 mg/ml, i.m.). A tracheostoma was placed to facilitate breathing and femoral artery was catheterized for blood pressure measurement. The femoral vein was catheterized for infusion of saline with 1% BSA (1 ml/hr/100g body weight). Following an abdominal incision, the left kidney
was exposed and immobilized in a kidney holder cup. The kidney orientation was positioned so that superficial tubules could be clearly visualized under the microscope (SZX16, Olympus, Tokyo, Japan). Tubular flow of a selected proximal tubule with multiple visible loops was obstructed with a grease block. Stop-flow pressure ($P_{sf}$) in proximal tubule upstream of the grease block was measured with servo-nulling method (Model 900A; World Precision Instruments, FL, USA). Micropipette for pressure measurement with a tip diameter at 2-5 µm was filled with 2 M KCl colored with 1% fast green. Proximal tubule distal to the grease block was perfused with artificial tubular fluid (ATF, containing 4 NaHCO$_3$, 5 KCl, 2 CaCl$_2$, 7 urea, 2 MgCl$_2$, 128 NaCl, and 1% fast green in mM; pH 7.4). $P_{sf}$ was measured when tubular perfusion rate was set at 0 nl/min and 40 nl/min for 3-5 min. The change of $P_{sf}$ ($\Delta P_{sf}$) was used as an index of TGF response mediated vasoconstriction in afferent arteriole. $\Delta P_{sf}$ was measured twice consecutively in each nephron in following three protocols in separate experiments: 1) 1$^{st}$ $\Delta P_{sf}$ was measured using ATF without glucose, then, 2$^{nd}$ $\Delta P_{sf}$ was measured while adding 16.7 mM glucose in ATF; 2) two $\Delta P_{sf}$ were measured using normal ATF without glucose; 3) 1$^{st}$ $\Delta P_{sf}$ was measured using normal ATF, and 2$^{nd}$ $\Delta P_{sf}$ was measured by adding 16.7 mM mannitol in ATF to determine whether the osmolarity has any effect on TGF.

**Measurement of TGF in vitro with microperfusion.** Rabbits were anesthetized with ketamine (50 mg/kg, i.m.) and kidneys were removed and sliced. Kidney slices were placed in ice cold Dulbecco’s modified Eagle’s medium-low glucose (DMEM) containing 5% bovine serum albumin (BSA). A single superficial intact glomerulus was microdissected together with adherent tubular segments consisting of the TAL, macula densa, and early distal tubule under a stereomicroscope (SMZ1500, Nikon, Yuko, Japan).
as described previously (Liu et al. 2004a). The dissected sample was transferred to a temperature-regulated chamber mounted on an inverted microscope (Axiovert 100TV, ZEISS, NY, USA). The glomerulus was held with glass pipette. The TAL was cannulated and perfused with another glass pipette with a low glucose macula densa solution containing: 10 HEPES, 1.0 CaCO$_3$, 0.5 K$_2$HPO$_4$, 4.0 KHCO$_3$, 1.2 MgSO$_4$, 5.5 glucose, 0.5 Na acetate, 0.5 Na lactate, and 10 NaCl in mM; pH 7.4. The high glucose macula densa solution contains the same ingredients as above except glucose was 16.7 mM.

TGF response in vitro was measured in isolated and double perfused JGAs as we reported previously (Song et al. 2015, Zhang et al. 2013). The distal tubule and Af-Art of the JGA from non-diabetic C57BL/6 mice were double perfused and the temperature in bath solution was gradually increased to 37°C for 30 min. TGF response was induced by increasing the NaCl concentration in tubular perfusate from 10 to 80 mM in low glucose concentration. TGF response was determined by the maximal constriction of the Af-Art. The tubular perfusate was switched back to 10 mM NaCl for 10 min, followed by switching to 80 mM NaCl solution containing 16.7 mM glucose. The TGF response was measured again. All solutions was adjusted to the same osmolarity with mannitol.

**Measurement of nitric oxide generation in macula densa.** For NO measurement, the macula densa was loaded with a fluorescent NO probe 4-amino-5-methylamino-2’, 7’-difluorofluorescein diacetate (DAF-2 DA; 10 μM plus 0.1% pluronic acid) from the tubular lumen for 30 min, then washed for 10 min with macula densa solution. DAF-2 was excited at 490 nm with a xenon light, and the emitted fluorescence was recorded at wavelengths of 510 to 550 nm. The rate of increase in fluorescent intensity of DAF-2 was used to determine NO generation by the macula densa as we
previously described (Fu et al. 2012, Wang et al. 2015). NO generation was measured for 5 min as basal condition when tubule was perfused with a low glucose macula densa solution. Then tubular perfusate was switched to a high glucose macula densa solution and perfused for 20 min. NO was measured again for another 5 min. Control experiments were performed without switching to a high glucose solution and using the same protocol and time course.

**Results**

Intravenous injection of glucose raised GFR in conscious mice. To determine whether intravenous injection of glucose alters GFR in normal mice, we measured GFR in conscious mice after infusion of glucose in C57BL/6 mice. GFR was 236±15.4 µl/min in all mice at basal. After an intravenous infusion of 50 µl 2 M glucose solution GFR was raised to 281±9.7 µl/min, which was a 19.1±3.5% increase compared to the baseline (*p<0.01 vs baseline, n=6, Figure 2). Intravenous infusion of same amount of isotonic saline did not significantly alter GFR, which was 239±10.2 µl/min after saline infusion.

To determine whether the effect of glucose infusion in GFR is dependent on NOS1 in the macula densa, we repeated the experiments in MD-NOS1KO mice. The basal GFR was 223±6.9 µl/min. After intravenous infusion of 50 µl 2 M glucose solution, GFR did not significantly increase (240±15.7 µl/min, n=6) in MD-NOS1KO mice.

We measured plasma glucose to demonstrate that glucose injection induces hyperglycemia. The blood glucose was 98±12 mg/dl at baseline in WT mice, it increased to 418±27 mg/dl after 3 min and returned to 137±36 mg/dl after 90 min of intravenous infusion of 50 µl 2 M glucose. In MD-NOS1KO mice, the blood glucose increased to
425±22 mg/dl from 106±20 mg/dl after 3 min and returned to 160±27 mg/dl after 90 min of acute glucose infusion (n=6, Figure 3).

**Figure 2. GFR after acute glucose infusion.**

GFR were measured and compared before and after an intravenous infusion of glucose (50 µl 2 M in saline) or 50 µl saline in conscious C57BL/6 WT and MD-NOS1KO mice. *p<0.01 vs baseline, n=6.

**Figure 3. The blood glucose levels after acute glucose infusion.**

The blood glucose were measured at 0, 3, and 90 min after an intravenous infusion of 50 µl 2 M glucose in WT and MD-NOS1KO mice. n=6.
Increased tubular glucose concentration blunted TGF response *in vivo*. To determine whether tubular glucose itself has any direct effect on TGF *in vivo*, we measured TGF response with micropuncture by adding glucose in ATF. The TGF response was measured twice consecutively in each nephron using ATF with and without glucose. When tubular perfusion rate of ATF was increased from 0 to 40 nl/min, $P_{sf}$ decreased from 39.7±2.1 to 34.2±2.9 mmHg. TGF response measured by $\Delta P_{sf}$ was 5.5±1.5 mmHg. After $P_{sf}$ returned to the baseline, ATF was added 16.7 mM glucose and $\Delta P_{sf}$ was measured again. $\Delta P_{sf}$ was reduced to 3.9±1.1 mmHg (from 40.2±1.7 to 36.3±2.5 mmHg) (*p*<0.05, n=4 mice/5 tubules, Figure 4A).

We repeated the experiments and measured two consecutive TGF responses without adding glucose as a control. There were no significant differences between the first and second TGF responses tubule was perfused with normal ATF only. $\Delta P_{sf}$ was 5.4±0.6 and 5.2±1.0 mmHg, respectively (Figure 4B, n=4 mice/6 tubules).

We did not adjust osmolality of ATF with glucose. To test whether osmolarity of the tubular fluid has any effect on TGF, we adjusted osmolarity of ATF without glucose from 293 mOsm to 310 mOsm (the same level of ATF with glucose) by adding 16.7 mM mannitol in ATF. $\Delta P_{sf}$ was 5.2±1.5 mmHg when normal ATF was used. After the tubular perfusate was switched to ATF with mannitol, $\Delta P_{sf}$ was 5.4±1.4 mmHg and did not significantly change (n=4 mice/5 tubules, Figure 4C).

To determine whether the glucose-induced inhibition on TGF response is mediated by NOS1 in the macula densa or due to the low NaCl concentration delivered to the macula densa, we repeated the experiments in MD-NOS1KO mice. $\Delta P_{sf}$ was 8.5±2.5 mmHg when tubule was perfused with normal ATF. After glucose (16.7 mM) was added...
to ATF, \( \Delta P_{sf} \) was 7.7±1.8 mmHg. There were no significant changes in TGF responses with and without glucose (\( n=4 \) mice/6 tubules, Figure 5A). In separate experiments, we repeated the protocol using normal ATF as a control. There were no significant changes between the first and second TGF responses when tubule was perfused with normal ATF. TGF responses of two consecutive measurements using ATF were 8.6±2.3 mmHg and 8.8±3.2 mmHg, respectively (\( n=3 \) mice/6 tubules, Figure 5B).

TGF responses were then compared between WT and MD-NOS1KO mice in response to increase of tubular perfusate glucose content. TGF response was attenuated from 5.5±1.5 mmHg to 3.9±1.1 mmHg after adding glucose in WT mice (*\( p<0.05 \) vs control, \( n=4 \), Figure 6). However, TGF response in MD-NOS1KO mice had no significant change in response to increase of glucose content in tubular perfusate.

**Figure 4. Effect of glucose on TGF response in vivo in WT mice.**

TGF response was measured with micropuncture by maximum change of \( P_{sf} \) when increasing tubular flow from 0 to 40 nl/min. **A**) Effect of adding glucose (16.7 mM) in ATF on TGF response. *\( p<0.05 \), \( n=4 \) mice/5 tubules. **B**) Time control of TGF responses with normal ATF only was used in tubular perfusate. \( n=4 \) mice/6 tubules. **C**) Effect of osmolality (from 293 to 310 mOsm) adjusted with mannitol on TGF response. \( n=4 \) mice/5 tubules.
TGF was measured in MD-NOS1KO mice in vivo by micropuncture. **A)** Effect of adding glucose (16.7 mM) in ATF on TGF response. n=3 mice/6 tubules. **B)** Time control of TGF responses with normal ATF only was used in tubular perfusate. n=4 mice/6 tubules.

*Figure 5. Effect of glucose on TGF response in vivo in MD-NOS1KO mice.*

The glucose-induced alterations of TGF response were compared between WT and MD-NOS1KO mice. *p<0.05 vs baseline.

*Figure 6. Effect of glucose on TGF response between WT and MD-NOS1KO mice.*
Increased glucose concentration at macula densa blunted TGF response in vitro. To determine whether tubular glucose itself has any direct effect on TGF in vitro, we measured TGF response with microperfusion in isolated and double perfused JGAs. TGF response was 3.8±0.2 µm when tubular glucose concentration was 100 mg/dl (low), and inhibited to 2.4±0.2 µm when tubular glucose concentration was increased to 300 mg/dl (high) (n=4, *p<0.05 vs low) (Figure 7). When the selective SGLT1 inhibitor KGA-2727 (10⁻⁶ M) was added to the tubular perfusate, the glucose-induced TGF inhibition was blocked (n=4). These data indicated that an increase in glucose concentration at the macula densa blunts the TGF response through a macula densa SGLT1-dependent mechanism.

Glucose directly enhanced NO generation by the macula densa. To determine whether glucose has any direct effect on NO generation, we isolated and perfused JGA loaded with DAF-2 DA. When tubule was perfused with low glucose solution, NO generation by the macula densa was 118.3±7.2 units/min. Then we switched tubular perfusate to a high glucose solution and perfused 20 min. NO generation increased by 78.4±9.1% (211±15.2 units/min) (*p<0.01 vs baseline, n=5, Figure 8).

In control experiments, we repeated the protocol but without switching to a high glucose solution. NO generation was 113.7±9.1 units/min at baseline and was 122.8±10.6 units/min after 20 min of tubular perfusion with low glucose solution (n=5).
An increase in glucose concentration at the macula densa blunts the TGF response through a macula densa SGLT1-dependent mechanism.

Figure 7. Glucose at the macula densa inhibits TGF by SGLT1.

An increase in glucose concentration at the macula densa blunts the TGF response through a macula densa SGLT1-dependent mechanism.
NO production induced by glucose was measured in macula densa with DAF-2 DA in isolated perfused JGA. NO generation was measured at the baseline and 20 min later when the tubules were perfused with either a low (n=5) or high glucose solution (n=5). *p<0.01 vs baseline.

**Figure 8. Effect of glucose on NO generation by the macula densa.**

Discussion

Present study provided evidences for a novel mechanism for diabetic hyperfiltration. We demonstrated that glucose directly enhances NOS1 activity and NO generation by the macula densa, which blunts TGF response and increases GFR.

Hyperfiltration is assumed to correlate with the development of diabetic nephropathy (O'Bryan & Hostetter 1997, Vora et al. 1992). The mechanisms leading to the increases of GFR in diabetes are not fully understood and several hypotheses have been proposed (Bank & Aynedjian 1993, Komers et al. 1994, Sabbatini et al. 1992,
Veelken et al. 2000), but no single factor that can fully account for the hyperfiltration of early diabetes. In the present study, we examined the direct effect of glucose on GFR in non-diabetic mice.

We first measured the changes in GFR in response to a bolus intravenous injection of glucose in normal C57BL/6 mice. We found that the GFR was raised by 19.1±3.5% in response to an acute glucose injection. However, the bolus injection of glucose did not increase GFR in MD-NOS1KO mice, in which NOS1 was specifically deleted from the macula densa (Lu et al. 2016), these results in the present study indicated that the glucose-induced elevations in GFR was mediated by NOS1 in the macula densa. Previous studies have shown that chronic hyperglycemia induced hyperfiltration. Hall et al. clearly demonstrated that chronic infusion of glucose significantly raised renal blood flow and GFR in anesthetized dogs. In addition, they also examined the possible role of TGF in hyperfiltration in nonfiltering kidneys (Woods et al. 1987). In nondiabetic humans, a 3-hour 5% glucose infusion caused a progressive increase in GFR (Appiani et al. 1990).

The mechanisms leading to the development of glomerular hyperfiltration with diabetes are not fully understood and several hypotheses have been proposed (Bank & Aynedjian 1993, Komers et al. 1994, Sabbatini et al. 1992, Veelken et al. 2000). Among them, the well-accepted theory is that enhanced SGLT activity lowers the luminal NaCl delivery to the macula densa, which blunts TGF response and contributes to the increased SNGFR (Vallon et al. 1995, Vallon et al. 1999, Vallon 2003). But it has not been clear whether glucose has any direct effect on TGF response. In present study, we measured TGF response in vivo with micropuncture. TGF response was inhibited by 29.1±2.6% when 16.7 mM glucose was included in tubular perfusate. To determine
whether the effect of glucose on TGF response was mediated on activation of SGLT or direct effect on the macula densa, we measured TGF response in the MD-NOS1KO mice. We did not find significant difference in TGF responses when tubular perfusate was with and without glucose. These data indicated that the acute effect of glucose was dependent on NOS1 activity in the macula densa.

Schnermann et al. demonstrated that TGF responsiveness is relatively constant when the perfusate osmolarity is varied from 130 to 400 mOsm (Briggs et al. 1980). However, more recent studies indicated that luminal osmolarity modulates macula densa cells volume, which may affect TGF responses (Blantz 2006, Komlosi et al. 2006, Liu et al. 2002b). In the present study, we did not adjust osmolarity in the ATF with glucose. To exclude the potential effect of osmolarity on TGF response in present study, we adjusted osmolarity of control ATF to the same level of ATF with glucose and did not find alterations in TGF response.

The data from MD-NOS1KO mice suggested that the effect of glucose was dependent on NOS1 activity in the macula densa. To determine whether glucose enhances NOS1 activity, we directly measured NO generation in the isolated perfused JGA with a NO fluorescent probe. We found that glucose quickly enhanced NO generation by the macula densa. Previous study indicated that high glucose increases the expression of endothelial nitric oxide synthase (eNOS) and production of NO in human aortic endothelial cells (Cosentino et al. 1997). In the kidney, it has been demonstrated that high glucose increases the NO production by upregulating inducible NOS (iNOS) through protein kinase C (PKC) pathway in cultured rat mesangial cells (Noh et al. 2002). Glucose has also been found to modulates NOS activity and NO generation
in renal cortex in which renal cortical slices were incubated at 37°C in solution containing concentrations of 5 or 20 mM glucose (Ishii *et al.* 2004). It has not been clear how glucose regulates NOS activity. The mechanisms need to be further determined.

In summary, we found that bolus injection of glucose raised GFR in conscious mice. Elevation of tubular glucose blunted TGF responsiveness and directly enhanced NO generation by the macula densa. We conclude that glucose contributes to the diabetic hyperfiltration by directly enhancing NOS1 activity and blunting TGF responsiveness in the macula densa. This new mechanism for diabetic hyperfiltration may provide a novel target for treatment for diabetic nephropathy.

**Project II: glucose dilates renal afferent arterioles via glucose transporter-1**

**Abstract**

Glomerular hyperfiltration often occurs during the early stage of diabetes. Acute glucose infusion in non-diabetic animals increases GFR. However, the results of afferent arteriolar responses to high glucose reported in previous microperfusion studies with non-preconstricted rabbit Af-Arts are controversial. The present confirmatory study determined the vasodilatory effect of increased glucose concentration in mouse Af-Arts with norepinephrine (NE) pre-constriction, as well as investigated the potential mechanisms. We isolated and microperfused the Af-Arts from non-diabetic C57BL/6 mice. The Af-Arts were preconstricted with NE (1 µM) for determination of the vascular dilation. When we switched the D-glucose concentration from low (5 mM) to high (30 mM), the pre-constricted Af-Arts significantly dilated by 37.8±7.1%, but L-glucose failed to dilate
the Af-Arts. GLUT1 mRNA was identified in microdisserted Af-Arts measured by RT-PCR. Nitric oxide (NO) production in Af-Art was measured using a cell-permeable fluorescent NO indicator 4,5-Diaminofluorescein diacetate. When the D-glucose concentration was switched from 5 to 30 mM, NO generation in Af-Art was significantly increased by 19.2±6.2% (84.7±4.1 to 101.0±9.3 units/min). L-glucose had no effect on the NO generation. A GLUT1 selective antagonist, STF-31, and a nitric oxide synthase inhibitor L-NG-Nitroarginine Methyl Ester blocked the high glucose-induced NO generation and vasodilation. In conclusion, we confirmed the vasodilatory effect of increased glucose level on Af-Art and demonstrated that the actions of high glucose in Af-Art is via GLUT1 and mediated by endothelium derived NO production.

**Introduction**

Diabetes mellitus is a major public health issue in the United States. According to the National Diabetes Statistic Report 2014, about 29.1 million or 9.3% of Americans have diabetes mellitus and it remains one of the leading causes of death in the United States. Metabolic disturbances, resulting from either the limitation in insulin production or the disruption of insulin sensitivity, lead to wide range of complications including heart disease, stroke, diabetic retinopathy and diabetic nephropathy (Brownlee 2005). Elevated GFR, also called glomerular hyperfiltration, in the early stage of diabetes mellitus was reported in about 70% of type 1 (Bank 1991, Levine 2008) and 50% of type 2 diabetic patients (Keller et al. 1996, Nelson et al. 1996, Vora et al. 1992), and has been recognized to promote the pathogenesis of diabetic nephropathy (Bank 1991, Keller et al. 1996, Levine 2008). The mechanism responsible for the diabetic glomerular hyperfiltration is
not fully elucidated and numerous factors have been implicated in the abnormality of glomerular hemodynamics (Helal et al. 2012, Tonneijck et al. 2017).

High blood glucose is the hallmark and most significant metabolic disturbance of diabetes mellitus (Giugliano et al. 2008). Intravenous infusion of glucose increases GFR in both normal people and diabetic patients (Brochner-Mortensen 1973a, Brochner-Mortensen 1973b, Christiansen et al. 1981), as well as in experimental animals (Noonan et al. 2001, Woods et al. 1987). As the primary pre-glomerular resistant vessels, renal Af-Arts play a critical role in control of glomerular hemodynamics. The glomerular hydrostatic pressure, the driving force of glomerular filtration, is reciprocally correlated with the resistance of Af-Arts (Gnudi et al. 2007, Loutzenhiser et al. 2004, Rosivall & Peti-Peterdi 2006). In the absence of systemic confounding factors, in vitro experiments with isolated and perfused rabbit Af-Arts have been applied to test the direct effect of high glucose on Af-Art, however, the results in these studies are controversial. Toma et al. indicated that the diameter of Af-Art was increased within 30 minutes of exposure to high glucose (Toma et al. 2008); whereas, a dose and time dependent constriction of Af-Art in response to high glucose was observed in another study by Arima et al. (Arima et al. 1995). Moreover, the vascular tone of isolated perfused arterioles is much lower than that in physiological condition (Hayashi et al. 1994, Ren et al. 2000a), therefore, it is hard to exactly determine the vasodilatory effect without the pre-constriction in these previous studies (Arima et al. 1995, Toma et al. 2008). On the other hand, mouse models, in particular, with the advantage of genetically modification, are much more broadly used than rabbit in in vivo studies on diabetes mellitus. Thus, despite of the larger technical challenge, the utilization
of mouse Af-Art is of greater significance in such *in vitro* experiment to investigate the
effect of glucose on vasoactivity.

Based on all above, the aim of this confirmatory study is to determine the
controversial outcomes of afferent arteriolar responses to high glucose in previous
studies. In the present study, the vasodilatory effect of high glucose was tested with
norepinephrine (NE) pre-constricted mouse Af-Arts. In addition, the potential molecular
mechanisms involved with endothelial NOS (NOS3)-derived nitric oxide (NO) and glucose
transporter 1 (GLUT1) were examined.

**Methods**

**Isolation and microperfusion of the Af-Art.** Similar methods as we previously
described (Lu *et al.* 2012, Lu *et al.* 2015, Lu *et al.* 2016) were used for isolation and
microperfusion of the Af-Arts. Briefly, the mice were anesthetized with inhaled isoflurane
(Butler Chemicals, Anaheim, CA). The kidneys were removed and sliced along the
corticomedullary axis. Slices were placed in ice-cold glucose Dulbecco’s modified Eagle’s
medium (DMEM). A single superficial intact glomerulus and its adherent Af-Art were
microdissected under a stereomicroscope (SMZ1500, Nikon, Yoko, Japan). The microdissected sample was transferred to a temperature-regulated chamber mounted on
an inverted microscope (Axiovert 100TV, ZEISS, Oberkochen, Germany) together with
DMEM containing 5 mM D-glucose (glucose). The glomerulus was held with a
micropipette and Af-Art was cannulated and perfused with another set of micropipettes.
The intraluminal pressure of the perfused Af-Art was maintained at 60 mmHg throughout
the experiment. The Af-Art was perfused with DMEM at 37°C for 30 minutes for
equilibration.
To determine whether glucose has a vasodilatory effect, Af-Art was preconstricted with norepinephrine (NE; 1 µM) added in lumen for 2-3 minutes. The baseline concentration of glucose in the DMEM was 5 mM. Then the Af-Art was perfused with NE plus high glucose (add 25 mM to 30 mM) or L-glucose (25 mM L-glucose with 5 mM glucose) for 5 min and the diameter of the Af-Art was measured. To determine the role of NO and GLUT1 in the glucose-induced vasodilatation, a NOS inhibitor L-NG-Nitroarginine Methyl Ester (L-NAME) or GLUT1 antagonist (STF-31) was used. The Af-Arts were pretreated with L-NAME (0.1 mM) or STF-31 (5 µM) for 30 minutes, and then the above experiments were repeated.

**NO measurement.** We measured NO production in the Af-Art using a cell-permeable fluorescent NO indicator 4,5-Diaminofluorescein diacetate (DAF-2 DA) as we described previously (Lu *et al.* 2016, Wang *et al.* 2016). Briefly, the Af-Art were loaded with 10 µM DAF-2 DA in 0.5% dimethyl sulfoxide plus 0.1% pluronic acid from the Af-Art lumen for 30 to 40 min, then washed for 10 min with DMEM. DAF-2 was excited at 490 nm with a xenon light, and the emitted fluorescence was recorded at wavelengths of 510 to 550 nm. Square-shaped regions of interest (ROIs) were set inside the cytoplasm of Af-Art and mean intensity within the ROIs was recorded every 5 s for 5 min. NO production was calculated as the percentage changes to the baseline of the DAF-2 fluorescence intensity.

**Reverse transcription–polymerase chain reaction (RT-PCR).** RT-PCR was used to determine whether GLUT-1 was expressed in the Af-Art. A single Af-Art was isolated from C57BL/6 mice and transferred into RLT buffer (RNeasy Mini Kit; Qiagen, Venlo, Netherlands) for RNA extraction as we previously described (Lu *et al.* 2012, Lu *et al.* 2012).
The time for dissection was less than 30 minutes after euthanatizing mice to avoid RNA degradation. Total RNA was reverse transcribed into cDNA with a reverse transcription system using oligo d (T) primer. The PCR was performed using a thermal cycler (MJ Mini; Bio-Rad, Hercules, CA) and PCR primer for GLUT1 was designed using Prime3Plus (GLUT1: 5’-CTGGCGGGAGACGCATAGTT-3’, 5’-CAAAGCGTGTTGAGTGTGGT-3). DNA was electrophoresed on a 1.5% agarose gel and images were captured using an image analysis system (ChemiDoc; Bio-Rad, Hercules, CA).

**Statistical analysis.** Data are presented as means ± SEM. The effects of interest was tested using ANOVA for repeated measures and a Student’s paired t-test or a post hoc Fisher's test when appropriate. Changes were considered to be significant if P < 0.05.

**Results**

**The vasodilatory effect of glucose on Af-Art.** To determine whether glucose dilates the Af-Art, we measured the effect of glucose in the preconstricted Af-Arts and compared with L-glucose. The basal diameter of the Af-Art was 9.85±0.37 μm and NE constricted the arterioles by 38.47±2.14 % (n=8; p<0.01 vs. baseline). When we increased the glucose concentration from 5 to 30 mM, the preconstricted Af-Art significantly dilated by 37.7±1.27 %. Af-Art diameter was increased from 6.05±0.21 to 8.34±0.35 mm (n=8; p<0.01 vs. low glucose). Then we repeated the above protocol and replaced glucose with L-glucose. L-glucose had no effect on the luminal diameter of NE-preconstricted Af-Art (Figure 9).
Glucose induced a vasodilation of preconstricted Af-Art by NE (A and B; *p<0.01 vs. NE). D-glucose dilated Af-Art by 37.8±7.1%, but L-glucose failed to dilate Af-Art (n=8) (C; *p <0.01 vs. L-glucose and Control).

GLUT1 is expressed in the Af-Art and facilitates the glucose-induced vasodilation. Expression of GLUT1 mRNA was measured by RT-PCR in isolated Af-Arts and clearly detected at 275 bp (n=4; Figure 10). To determine whether glucose dilates the Af-Art via activation of GLUT1, we used a GLUT1 antagonist STF-31. The Af-Art diameter was 9.07±0.12 μm at baseline and STF-31 had no effect on the baseline Af-Art diameter. Then, NE constricted the Af-Arts by 55.76±0.39 % (n=7; p<0.01 vs. baseline). In the presence of STF-31, an increase in glucose concentration had no effect on the diameter of the Af-Art (Figure 11), indicating that glucose-induced vasodilation is mediated by GLUT1 in the Af-Art.
Figure 10. The expression of GLUT1 receptor in the Af-Art.


Figure 11. STF-31 inhibits glucose induced vasodilation.

The vasodilative effect of D-glucose on STF-31 pretreated Af-Art were measured. D-glucose failed to dilate NE preconstricted Af-Art in present of STF-31 (n=7).

L-NAME inhibits glucose induced vasodilation. To determine whether glucose dilates the Af-Art via NO, we used a non-selective NOS inhibitor L-NAME. The Af-Art diameter was 9.17±0.27 µm at baseline and decreased to 8.76±0.30 µm in the presence
of L-NAME. Then the Af-Art diameter was constricted to 56.9±1.6% of baseline with NE (p<0.01 vs. baseline). In the presence of L-NAME, increase in glucose concentration did not dilate the Af-Art (n=5; Figure 12), demonstrating that glucose dilates the Af-Art mediated by NO.

![Graph showing luminal diameter changes](image)

**Figure 12. L-NAME inhibits glucose induced vasodilation.**

The vasodilative effect of D-glucose on L-NAME pretreated Af-Art were measured. D-glucose failed to dilate NE preconstricted Af-Art in present of L-NAME (n=5).

**Glucose enhances NO generation of Af-Art.** To determine whether glucose has any effect on NO generation, we loaded the perfused Af-Art with DAF-2 DA. NE constricted the Af-Art, but did not significantly alter NO generation. NO production in the Af-Art was increased by 19.2±6.2% in response to an increase in glucose concentration (n=6; p<0.05 vs. baseline). L-glucose had no effect on NO generation. Pretreated with STF-31 and L-NAME blocked the glucose-induced NO generation (Figure 13A-B).
Representative images (A) demonstrated NO generation after D-glucose and L-glucose treatment. D-glucose enhanced NO generation of Af-Art (n=6) (B; \*p<0.01 vs. other groups).

Figure 13. Glucose enhances NO generation of Af-Art.
Discussion

The results of afferent arteriolar responses to high glucose reported in previous *in vitro* microperfusion studies with non-preconstricted rabbit Af-Arts are controversial. The present confirmatory study determined the vasodilatory effect of increased glucose concentration in isolated and perfused mouse Af-Arts with NE pre-constriction. We found that high glucose dilated the Af-Arts, which was mediated by NOS3 via GLUT1.

Increased GFR is a major characteristic in the early stage of diabetes mellitus and has been considered to contribute to the diabetic glomerular injury. However, the mechanism responsible for diabetic glomerular hyperfiltration is not completely understood. As the hallmark and most significant metabolic disorder of diabetes mellitus, high blood glucose is believed to be implicated in the abnormality of glomerular hemodynamics (Giugliano *et al.* 2008). Intravenous infusion of glucose increases GFR in both clinical patients (Brochner-Mortensen 1973a, Brochner-Mortensen 1973b, Christiansen *et al.* 1981) and experimental animals (Noonan *et al.* 2001, Woods *et al.* 1987). Renal Af-Arts, the primary preglomerular resistant vessels, play a critical role in regulation of glomerular hemodynamics. GFR is negatively related with the resistance of Af-Arts (Gnudi *et al.* 2007, Loutzenhiser *et al.* 2004, Rosivall & Peti-Peterdi 2006).

The vascular response of Af-Art to increased glucose concentration was previously examined in two microperfusion studies with non-preconstricted rabbit Af-Arts, however, the results are inconsistent. In the study by Toma *et al.*, 30 minutes of exposure to high glucose dilated Af-Art (Toma *et al.* 2008); whereas, the other study by Arima *et al.* showed a dose and time dependent vasoconstriction of Af-Art in response to increased glucose concentration (Arima *et al.* 1995). The controversial outcomes might be due to the largely
reduced vascular tone in the isolated and perfused Af-Arts (Hayashi et al. 1994, Ren et al. 2000a). It is indispensable to pre-constrict the Af-Arts with NE before testing vasodilatory responses. Thus, in the present study, we determined the vasodilatory effect of increased glucose concentration in isolated and perfused mouse Af-Arts with NE pre-constriction, and our results demonstrated that an increase in glucose level dilated Af-Art. Additionally, since the osmolality could affect vascular reactivity (Ezimokhai & Osman 1998, Zakaria et al. 2005), L-glucose, the enantiomer of D-glucose, was utilized to balance the osmolality as control. Our results demonstrated that same concentration of L-glucose had no significant effect on the diameter of Af-Art, which indicates that the high glucose induced vasodilation of Af-Arts was independent of the alteration in osmolality.

For most mammalian cells, uptake of glucose across cell membrane is a process of facilitated diffusion, in which glucose is passively transported following its concentration gradient without energy consumption but mediated via a specific family of integral transmembrane proteins, namely the glucose transporters (Augustin 2010, Thorens & Mueckler 2010, Zhao & Keating 2007). Fourteen glucose transporter isoforms have been identified with cell or tissue specific distributions throughout the body (Olson & Pessin 1996, Wood & Trayhurn 2003, Zhao & Keating 2007). In vasculature, GLUT1 is recognized as the primary isoform responsible for glucose transport in endothelium (Abdul Muneer et al. 2011, Kaiser et al. 1993, Peti-Peterdi 2010, Sone et al. 2000) and vascular smooth muscle (Kaiser et al. 1993, Lin et al. 2013, Pyla et al. 2013). Whether the Af-Arts express GLUT1 has not been investigated. We isolated the Af-Art and then examined the mRNA expression of GLUT1 with RT-PCR. We demonstrated that GLUT1 mRNA was expressed in Af-Arts. With the method of microdissection of the Af-Art, we are
certain that what we measured was exclusively from the Af-Art and there was no contamination from the other arteries or tubules. However, it is difficult to examine the protein level due to the limited amount of samples collected with this method. To further determine the role of GLUT1 in high glucose-induced vasodilation of Af-Arts, we utilized a GLUT1-selective inhibitor STF-31. We found that inhibition of GLUT1 blocked the vascular reactivity to the increased glucose concentration, indicating that the vasodilatory effect of the glucose was mediated via GLUT1.

It is well known that endothelial-derived NO causes vascular smooth muscle relaxation via activation of soluble guanylate cyclase, which reduces vascular resistance of Af-Arts and increases GFR (Bachmann & Mundel 1994, Edwards & Trizna 1993, Wilcox 2005). In the present study, we demonstrated that NOS inhibition completely blocked the vascular response to increased glucose concentration in Af-Arts, which indicates that the high glucose-induced vasodilation of the Af-Arts is mediated by NO. These results are consistent with the previous report that L-NAME abolished the vasodilatory effect of application of high glucose in non-preconstricted rabbit Af-Arts (Toma et al. 2008).

Then, we directly measured the NO production in isolated perfused Af-Arts by using the fluorescent indicator DFA-2 DA. We demonstrated that high D-glucose but not L-glucose significantly increased the generation of NO in Af-Arts, accompanied by the increase in lumenal diameter; administration of GLUT1 inhibitor completely inhibited the high-glucose induced NO release. These findings suggest that hyperglycemia elevates the intracellular glucose level in the endothelial cells of renal Af-Arts via GLUT1, which stimulates NOS3 activity and increases the endothelium derived NO production. Similar
to our findings, previous studies also demonstrated that high glucose increased the NOS3 expression and NO generation in human aortic endothelial cells (Cosentino et al. 1997, Srinivasan et al. 2004) and mouse microvascular endothelial cells (Ding et al. 2007).

The mechanism by which high glucose increases NOS3 activity has not been fully elucidated. Several studies explored the potential signaling pathway, which involves the local accumulation of succinate and activation of the kidney-specific G protein-coupled metabolic receptor GPR91 (Peti-Peterdi 2010, Toma et al. 2008), the activation of a A2a purinoceptor (San & Sobrevia 2006) and Akt1-mediated phosphorylation of NOS3 (Dimmeler et al. 1999, Huang & Sheibani 2008).

Additionally, in the present study, we demonstrated that the application of NE per se did not increase the NO release, which indicates that the NOS3 activity of Af-Arts was not significantly influenced by NE stimulation. This finding is consistent with the result of a previous study by Patzak et al. that L-NAME pretreatment did not significantly change the sensitivity of the Af-Arts to NE (Patzak et al. 2004).

In summary, this confirmatory study determined the previous controversial outcomes of afferent arteriolar responses to high glucose. We demonstrated that increased glucose concentration dilates Af-Art through GLUT1 and mediated by the endothelium derived NO production. The results of present study not only confirm the significant role of high glucose induced vasodilation of Af-Art in the renal hemodynamic changes of diabetes, but also provide a potential therapeutic target, GLUT1 in Af-Arts for the prevention of glomerular hyperfiltration.
Chapter Three: Hypertension in Diabetes

Project I: deletion of NOS1 in macula densa induces hypertension in diabetes

Introduction

More than 30 million of Americans – 9.4% of the population have diabetes. Total annual medical costs for people with diabetes is over $245 billion (Patzak et al. 2004). The rate of hypertension is more than 2-fold higher in patients with diabetes than those without the disease (Colosia et al. 2013, Gillespie & Hurvitz 2013). Diabetic patients with hypertension exhibit an increased risk for diabetic complications, such as coronary heart disease, stroke, peripheral vascular disease, retinopathy and nephropathy (Estacio & Schrier 2007, Haffner et al. 1998, Sowers et al. 2001). However, the mechanisms underlying the high prevalence of hypertension in diabetic patients are not fully elucidated.

An increase in GFR or glomerular hyperfiltration has been observed in about 70% of type 1 (Bank 1991, Levine 2008) and 50% of type 2 diabetic patients (Keller et al. 1996, Vora et al. 1992) and associated with an increased risk for diabetic nephropathy and worse prognosis (Abdi et al. 2012, Helal et al. 2012, Persson et al. 2010). Nevertheless, the mechanism for glomerular hyperfiltration has not been fully clarified and the link between GFR and hypertension in diabetes remains elusive. Several mechanisms have been implicated in diabetic glomerular hyperfiltration, including the TGF control of GFR. A TGF response is initiated by an increase in NaCl delivery to the macula densa, which
promotes the release and formation of ATP and/or adenosine that constricts the afferent arteriole, and thus the TGF induces a tonic inhibition of SNGFR. In the macula densa, neuronal nitric oxide synthase (NOS1) is the major NOS isoform and nitric oxide (NO) is primarily generated by the NOS1 splice variant β. Macula densa NO blunts the TGF response and NOS1 expression is increased in the diabetic kidney. Therefore, NOS1 in the macula densa may contribute to glomerular hyperfiltration in diabetes and we hypothesized that macula densa NO attenuates TGF response and contributes to diabetic glomerular hyperfiltration. On the other hand, insufficient macula densa NO generation in the setting of hyperglycemia limits the increase in GFR and promotes hypertension in diabetes.

**Methods**

**Generation of type 2 diabetic db/db-MD-NOS1KO mice.** Type 2 diabetic db/db-MD-NOS1KO mice, a type 2 db/db mouse line with macula densa specific NOS1 deletion were generated. MD-NOS1KO (NOS1flox/flox; NKCC2cre) mice with heterozygotes db/+ (Leprdb/+) were crossed, since homozygotes (Leprdb) were infertile. The db/db-MD-NOS1KO mice were db/db (Leprdb) with NOS1flox/flox; NKCC2cre. Littermate db/db mice will be used as diabetic controls, littermate db/+ (Lepr^{db/+}) and MD-NOS1KO mice will be used as non-diabetic controls.

**Measurement of nitric oxide generation in macula densa.** Mice were anesthetized with ketamine (50 mg/kg, i.m.) and kidneys were removed and sliced. Kidney slices were placed in ice cold Dulbecco’s modified eagle’s medium-low glucose (DMEM) containing 5% bovine serum albumin (BSA). A single superficial intact glomerulus was microdissected together with adherent tubular segments consisting of the
TAL, macula densa, and early distal tubule under a stereomicroscope (SMZ1500, Nikon, Yuko, Japan) as described previously (Liu et al. 2004a). The dissected sample was transferred to a temperature-regulated chamber mounted on an inverted microscope (Axiovert 100TV, ZEISS, NY, USA). The glomerulus was hold with glass pipette. The TAL was cannulated and perfused with another glass pipette with a low glucose macula densa solution containing: 10 HEPES, 1.0 CaCO\textsubscript{3}, 0.5 K\textsubscript{2}HPO\textsubscript{4}, 4.0 KHCO\textsubscript{3}, 1.2 MgSO\textsubscript{4}, 5.5 glucose, 0.5 Na acetate, 0.5 Na lactate, and 10 NaCl in mM; pH 7.4. For NO measurement, the macula densa was loaded with a fluorescent NO probe 4-amino-5-methylamino-2', 7'-difluorofluorescein diacetate (DAF-2 DA; 10 μM plus 0.1% pluronic acid) from the tubular lumen for 30 min, then washed for 10 min with macula densa solution. DAF-2 was excited at 490 nm with a xenon light, and the emitted fluorescence was recorded at wavelengths of 510 to 550 nm. The rate of increase in fluorescent intensity of DAF-2 was used to determine NO generation by the macula densa as we previously described (Fu et al. 2012, Lu et al. 2016, Wang et al. 2015).

**Measurements of GFR in conscious mice.** GFR was measured by the clearance of plasma FITC-inulin with a single bolus injection in conscious mice. Mice were lightly anesthetized with isoflurane and injected with the FITC-inulin solution (3.74 μl/g body weight) through the penile vein. Blood (10 μl) was collected into heparinized capillary tubes through the tail vein at 3, 7, 10, 15, 35, 55, 75, and 90 minutes after inulin injection. The blood samples were centrifuged at 8000 rpm for 5 minutes at 4˚C. Plasma (1 μl) was collected from each sample. FITC-inulin concentration of the plasma was measured using a plate reader (Cytation3, BioTek, VT) with 485-nm excitation and 538-nm emission. GFR were calculated with GraphPad Prism 6 (GraphPad Software).
**Implantation of transmitters.** Telemetry transmitters (PA-C10; Data Sciences International, MN, USA) were implanted to measure MAP. Mice were anesthetized with inhaled isoflurane (Butler chemicals, UK) and placed on the temperature-controlled operating table (03-02; Vestavia Scientific, AL, USA). The left carotid artery was exposed by a small incision in the middle of the neck. The pressure catheter was implanted in the left carotid artery, and the telemetric device was placed subcutaneously in the right ventral flank of the mice. MAP was recorded for 10 seconds every 2 min for 4 hours from 1:00 PM to 5:00 PM starting from 7 days after the transmitter implantation.

**Light microscopy.** The kidneys were harvested and fixed in 4% paraformaldehyde solution. Fixed kidney tissues were embedded in paraffin, and 2 µm kidney tissue slices were cut and stained with Periodic Acid Schiff (PAS) or Jone’s sliver. Ten randomly chosen fields were captured under 1000x magnification and the degree of glomerular injury was quantified by the percentage of mesangial expansion (<25%, 25–50%, 50–80%, >80%) (Tervaert et al. 2010). All morphometric analyses were performed in blinded manner.

**Transmission electron microscopy (TEM).** Briefly, with an incision to the right atrium of the heart, 1M phosphate buffered saline (PBS) solution with 0.8 Units/mL heparin was perfused via the left ventricle to flush the blood. Small tissue pieces (approximately 1 mm³) of renal cortex were cut and fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) overnight at room temperature. The tissue pieces were rinsed with 0.1 M sodium cacodylate buffer (pH 7.4) and treated with 1% osmium tetroxide for 2 h. Then the tissue pieces were dehydrated with a series of graded ethanol, sequentially infiltrated with 2:1, 1:1, 1:2 mixtures of acetone and resin, and embedded in
100% resin. Thin sections (approximately 90-100 nm) were cut and examined with transmission electron microscope (JEM-1400Plus) at USF Health Lisa Muma Weitz Laboratory. The thickness of glomerular basement membrane (GBM) was defined by the shortest distance between the endothelial plasma membrane and the plasma membrane of the podocyte foot processes. The mean GBM thickness of each glomerulus was calculated as described (Guo et al. 2005, Ramage et al. 2002). The podocyte foot process was detected and the average width of foot process in each glomerulus was determined as described (Gao et al. 2010, van den Berg et al. 2004).

Results

To determine whether NOS1 was deleted from the macula densa in db/db-MD-NOS1KO mice, we measured macula densa NO generation with DAF-2DA in isolated perfused JGAs. As demonstrated in Figure 14, macula densa NO generation was significantly increased in db/db mice compared with non-diabetic db/+ mice. In the db/db-MD-NOS1KO mice, macula densa NO generation was diminished. These data indicate that NO generation by the macula densa is enhanced in type 2 db/db diabetic mice compared with non-diabetic controls, and NOS1 has been effectively deleted from the macula densa in db/db-MD-NOS1KO mice.
NO generation by the macula densa was enhanced in db/db mice compared with db/+ and significantly diminished in the db/db-MD-NOS1KO mice (*p<0.05 vs. db/+; #p<0.01 vs. db/db).

To determine whether macula densa NOS1 contributes to glomerular hyperfiltration in type 2 diabetes, we measured GFR with FITC-inulin in conscious db/db-MD-NOS1KO mice and compared with age-matched diabetic db/db and non-diabetic db/+ controls. As depicted in Figure 15, GFR increased by 58.8±6.5% in db/db mice compared with non-diabetic controls. Deletion of NOS1 reduced GFR by 24.2±3.5% in db/db-MD-NOS1KO mice compared with db/db mice. These data suggested that macula densa NOS1 contributes to the development of glomerular hyperfiltration in type 2 diabetes.
Figure 15. GFR.

GFR increased by 58.8±6.5% in db/db mice compared with db/+ mice. Deletion of NOS1 reduced GFR by 24.2±3.5% in db/db-MD-NOS1KO mice compared with db/db mice (*p<0.01 vs. db/+; #p<0.05 vs. db/db).

Figure 16. MAP.

MAP was increased to 124±8.1mmHg in db/db-MD-NOS1KO mice compared with db/+ and db/db mice (*p<0.05 vs. db/+ and db/db).
To determine whether deletion of macula densa NOS1 promotes the development of hypertension in diabetes, MAP was measured for 7 days in db/db-MD-NOS1KO mice and littermate db/db and db/+ mice. Average MAP over the 7-day period was 95±3 mmHg in db/+ mice (n=4), 103±4 mmHg in db/db mice (n=3) and increased to 124±6 mmHg in db/db-MD-NOS1KO mice (n=4) (Figure 16).

We examined the histology of diabetic kidney injury with light microscope and TEM in littermate non-diabetic db/+, diabetic db/db and db/db-MD-NOS1KO mice at age of 24 weeks. As shown in Figure 17A, kidney slices were stained with PAS and Jone’s silver. Non-diabetic db/+ mice exhibited basically normal structure with no mesangial expansion. Mesangial expansion (typical changes are indicated by red arrows) was observed in glomeruli in db/db mice and more severe in the db/db-MD-NOS1KO mice with enlarged glomeruli (Figure 17B). Figure 18A shows TEM images. Glomerular basement membrane (GBM) (red arrows) and podocyte foot processes (white arrows) are basically normal in db/+ mice, with mild to moderate damage in db/db mice and severe damage in the db/db-MD-NOS1KO mice, reflected by the thickness of GBM (Figure 18B) and width and density of foot process (Figure 18C and 18D). These data indicated that db/db-MD-NOS1KO mice with hypertension exhibit more severe diabetic kidney injury.
Non-diabetic db/+ mice exhibited basically normal structure with no mesangial expansion. The db/db-MD-NOS1KO mice exhibited more severe glomerular injury compared with db/db mice.

Figure 17. Light microscopy.
The db/db-MD-NOS1KO mice exhibited more severe diabetic kidney injury compared with db/db mice, reflected by the thickness of GBM and width and density of foot process.

**Discussion**

In the present study, we found that in diabetes, upregulated NOS1 activity blunts the TGF response and promotes glomerular hyperfiltration; conversely, inhibition macula densa NOS1 induces hypertension by limiting the rise in GFR.

The pathogenesis of glomerular hyperfiltration in diabetes has not been fully understood and several mechanisms have been proposed, including primary vascular and primary tubular mechanisms. According to the vascular hypothesis, hyperfiltration results from imbalance between vasoconstrictive factors (Ang II, thromboxane A2, endothelin-1 and its ETA receptor, and reactive oxygen species) and vasodilatory factors (NO, prostanoids, kallikrein-kinin, atrial natriuretic peptide, Ang 1-7 and hyperinsulinemia)
The tubular hypothesis proposes that tubular growth and the sodium-glucose cotransporter-2 (SGLT2) enhance proximal tubular reabsorption, which reduces NaCl delivery to the macula densa and increases GFR via the TGF mechanism (Thomson et al. 2004b, Thomson et al. 2012, Vallon et al. 1999). Even though many studies assessed the renal NO production and TGF response in diabetes (Komers et al. 2000a, Levine et al. 2006, Thomson et al. 2004a), whether macula densa NOS1 is a causal factor for glomerular hyperfiltration remains elusive (Persson et al. 2010, Tonneijck et al. 2017, Vallon et al. 1999). In the present study, we demonstrated that GFR increased in db/db mice compared with non-diabetic controls and the deletion of NOS1 reduced GFR in db/db-MD-NOS1KO mice compared with db/db mice. These data suggested that macula densa NOS1 contributes to the development of glomerular hyperfiltration in type 2 diabetes. We expect that hyperglycemia increases glucose filtration and enhances SGLT2-mediated Na-glucose reabsorption, which decreases NaCl delivery to the macula densa and inhibits the vasoconstrictor TGF tone (Nordquist et al. 2009, Thomson et al. 2001, Thomson et al. 2004b, Vallon et al. 1999). At the same time, the increased glucose concentration at the macula densa enhances NOS1 expression and activity and further reduces the vasoconstrictor TGF tone. These two signaling pathways additively promote hyperfiltration in diabetes.

The rate of hypertension is more than twice in diabetic patients than in the non-diabetic population. Diabetic patients with hypertension exhibit an increased risk of cardiovascular disease and exacerbation of other diabetic complications. But the link between glomerular hyperfiltration and hypertension in diabetes has not been fully
elucidated. In the present study, we demonstrated that MAP was significantly increased in db/db-MD-NOS1KO mice compared with db/db mice. These data indicated that deletion of macula densa NOS1 promotes the development of hypertension by limiting the rise in GFR.

Our result is not contradictory to the extensive literature about glomerular hyperfiltration, which is considered as a risk factor for diabetic nephropathy and worse prognosis (Bank 1991, Helal et al. 2012, Keller et al. 1996), since it seems per our data that glomerular hyperfiltration is protective in diabetes. Actually, the present study in particular applies to diabetic patients whose blood glucose is not well-controlled and consequently have increased tubular glucose concentration at the macula densa. For diabetic patients with well-controlled blood glucose, the tubular glucose level at the macula densa is expected to be minimal, by which the stimulatory effect on macula densa NOS1 is minimum. Therefore, these patients, with either normal or reduced macula densa NOS1 expression, would be expected to exhibit normal GFR and blood pressure with less diabetic complications including nephropathy. In accordance, clinical studies have demonstrated the significant role of blood glucose in hyperfiltration: glomerular hyperfiltration in diabetes improves after effective insulin therapy, but worsens with poor plasma glucose control (Bank 1991, Keller et al. 1996). The present study examined the determinants of GFR and blood pressure and their interactions in the early stage of diabetes, i.e. before structural changes due to diabetic kidney injury induce secondary effects on GFR and blood pressure. Even though we focus on the effect of glucose itself on macula densa NOS1 activity, we are aware of the complexity of the pathophysiological
mechanisms in diabetes, which affect many systems and organs (Lehrke & Marx 2017, Vallon & Thomson 2012).

In summary, we demonstrated that insufficient NO generation by the macula densa induces hypertension in the presence of hyperglycemia in diabetes by lowering GFR. The present study establishes a decisive role of macula densa NOS1 as key determinants of diabetes-induced glomerular hyperfiltration as well as hypertension. Changes in macula densa NOS1 activity may also determine the switch from diabetic hyperfiltration to hypertension, and thus provide a novel therapeutic target for diabetes-induced hypertension and its complications.

Project II: enhanced hemodynamic responses to angiotensin II in diabetes are associated with increased expression and activity of AT1 receptors in the afferent arteriole

Abstract

The prevalence of hypertension is about 2-fold higher in diabetic than in non-diabetic subjects. Hypertension aggravates the progression of diabetic complications, especially diabetic nephropathy. However, the mechanisms for the development of hypertension in diabetes have not been elucidated. We hypothesized that enhanced constrictive responsiveness of renal afferent arterioles (Af-Art) to angiotensin II (Ang II) mediated by Ang II type 1 (AT1) receptors contributes to the development of hypertension in diabetes. In response to an acute bolus intravenous injection of Ang II, alloxan-induced
diabetic mice exhibited a higher mean. Mean arterial pressure (MAP) (119.1±3.8 mmHg vs.106.2±3.5 mmHg) and a lower renal blood flow (0.25±0.07 mL/min vs.0.52±0.14 mL/min) compared with non-diabetic mice. In response to chronic Ang II infusion, the MAP measured with telemetry increased by 55.8±6.5 mmHg in diabetic mice, but only by 32.3±3.8mmHg in non-diabetic mice. The mRNA level of AT1 receptor increased by about 10 folds in isolated Af-Art of diabetic mice compared with non-diabetic mice, whereas Ang II type 2 (AT2) receptor expression did not change. The Ang II dose-response curve of the Af-Art was significantly enhanced in diabetic mice. Moreover, the AT1 receptor antagonist, losartan, blocked the Ang II-induced vasoconstriction in both diabetic mice and non-diabetic mice. In conclusion, we found enhanced expression of the AT1 receptor and exaggerated response to Ang II of the Af-Art in diabetes, which may contribute to the increased prevalence of hypertension in diabetes.

**Introduction**

Diabetes mellitus and hypertension are two major health issues in the United States. The prevalence of diabetes was 9.3% as of 2012, and hypertension affects about one-third of the adult population (Ong et al. 2008). Diabetes and hypertension are interrelated and coexist (Sowers et al. 2001). The prevalence of hypertension in diabetic subjects is approximately twice that of the non-diabetic population (Epstein & Sowers 1992, Klein et al. 1984, Pell & D'Alonzo 1967, Teuscher et al. 1989, Vaishnava & Bhasin 1969). The presence of hypertension in diabetic patients further exacerbates their morbidity and mortality (Epstein & Sowers 1992, Gillow et al. 1999, Klein & Klein 2002). However, the pathophysiological mechanisms for the development of hypertension in diabetes have not been completely elucidated.
The renin-angiotensin system (RAS) plays an essential role in regulating blood pressure and is one of the most important therapeutic targets for hypertension (Crowley et al. 2005, Hall 1991a, Hall 1991b). RAS blockers, such as angiotensin-converting enzyme inhibitors and angiotensin receptor blockers, have been recognized as the first-line treatment with additional beneficial effects for hypertensive patients with diabetes, which suggests the involvement of RAS in the development of hypertension in diabetic subjects (Arauz-Pacheco et al. 2004, Kirpichnikov & Sowers 2002, Reboldi et al. 2009). Although plasma renin was suppressed (Christlieb et al. 1976, Fernandez-Cruz, Jr. et al. 1981, Price et al. 1999) or normal (Beretta-Piccoli et al. 1981, Luik et al. 2003) in diabetic patients, the responsiveness of blood pressure to angiotensin II (Ang II) was demonstrated to be exaggerated in normotensive and mildly hypertensive patients with Type I diabetes (Drury et al. 1984, Weidmann et al. 1979). However, the mechanism of the enhancement in blood pressure response to Ang II remains elusive.

A previous study with kidney cross-transplantation between wild-type and Ang II type 1 (AT1) receptor-deficient mice examined the action of AT1 receptors in the kidney. They demonstrated the predominant role of the renal AT1 receptors in the development and maintenance of Ang II-dependent hypertension (Crowley et al. 2006). The afferent arterioles (Af-Arts) are the major resistance vessels in the kidney and play a critical role in regulating renal hemodynamics and blood pressure (Dilley et al. 1984, Gothberg et al. 1979, Knox et al. 1974, Loutzenhiser et al. 2006). Therefore, we hypothesized in the present study that the increased constrictive responsiveness of the Af-Art to Ang II mediated by the AT1 receptor contributes to the enhanced response of renal hemodynamics to Ang II and the development of hypertension in diabetes. We examined
the responses of blood pressure and renal hemodynamic changes to acute and chronic Ang II infusion in diabetic mice and measured the Ang II dose-response curve in the isolated perfused Af-Art.

**Methods**

All studies were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of South Florida, College of Medicine.

**Induction of diabetes.** C57BL/6 mice (male, 13-15 weeks old) were purchased from Jackson Laboratory. The mice were housed at 23±1.0°C on a 12:12-h light-dark cycle with lights on at 6 AM and free access to standard chow and water. Diabetes was induced by an intravenous injection of alloxan (55 mg/kg in 100 µl saline) through the penile vein after overnight fasting. Blood glucose was measured twice a week starting 3 days after alloxan injection (Figure 19). Mice with 300-500 mg/dL blood glucose levels were used in the present study. The mice with high blood glucose level (>500 mg/dL) were treated with insulin (0.5 U/kg) to maintain the blood glucose at 300-500 mg/dL. The mice with an injection of 100 µl saline through the penile vein after overnight fasting served as non-diabetic animals. Measurement of blood glucose of the non-diabetic mice

![Figure 19. The change of blood glucose after alloxan injection.](image)

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was the same as in the diabetic mice. All experiments were performed at 4 weeks after alloxan or saline injection.

**Mean arterial pressure (MAP) and renal hemodynamic responses to acute Ang II injection.** Mice were anesthetized with pentobarbital (50 mg/kg I.P.) and placed on a temperature-controlled operating table (Vestavia Scientific, Vestavia Hills, AL) to maintain body temperature at 37±1.0°C. The carotid artery was catheterized for blood pressure measurement with a PowerLab (ADInstruments, Boulder, CO). Following an abdominal incision, the left renal artery was carefully separated from the vein, and a perivascular flow probe (Transonic Systems, Ithaca, NY) was placed around the left renal artery. The probe was then stabilized by a micromanipulator. Renal blood flow (RBF) measured with the flow probe was recorded via the PowerLab. After 30 min equilibration, MAP and RBF were measured for 5 min as a baseline. Then a bolus injection of 1 ng/kg Ang II (dissolved in 20 μl of 0.9% saline) was given through a penile vein (Bek et al. 2006). MAP and RBF were recorded for 15 min. RVR was calculated as the renal artery pressure (same as MAP) divided by RBF.

**MAP response to chronic Ang II infusion.** Telemetry transmitters (PA-C10; Data Sciences International, MN, USA) were implanted for measurement of MAP in conscious mice as we previously described (Lu et al. 2016, Zhang et al. 2014). Briefly, the mice were anesthetized with inhaled isoflurane (Butler chemicals, UK) and placed on the temperature-controlled operating table. The left carotid artery was exposed by a small incision in the middle of the neck. The pressure catheter was implanted in the left carotid artery, and the telemetric device was placed subcutaneously in the right ventral flank of the mice. MAP was most stable from 1:00 PM to 5:00 PM during a 24-hour period, so the
MAP and heart rate were recorded for 10 seconds every 2 min for 4 hours from 1:00 PM to 5:00 PM starting from 7 days after the transmitter implantation.

Five days after baseline MAP measurement, micro-osmotic pumps (MODEL 1002; ALZET, CA, USA) were subcutaneously implanted as described previously (Zhang et al. 2014). Briefly, micro-osmotic pumps were filled with Ang II (600 ng/kg/min) and incubated in sterile saline at 37 °C overnight to reach steady state before implantation. Mice were anesthetized with isoflurane. A small incision was made in the skin between the scapulae, and a small pocket was formed by separating the subcutaneous connective tissues from the skin. The micro-osmotic pump was inserted into the pocket, and the wound was sutured. Fourteen days after implantation, the micro-osmotic pumps were removed from the animals and the new pumps filled with Ang II were subcutaneously implanted.

**Isolation and microperfusion of Af-Art.** The isolation and micro-perfusion of the Af-Arts were the same as we described previously (Lu et al. 2012, Lu et al. 2015, Lu et al. 2016). Briefly, the mice without Ang II infusion were anesthetized with inhaled isoflurane and the kidneys were removed and sliced. The kidney slices were placed in ice-cold Dulbecco’s modified eagle’s medium (DMEM). A single superficial intact glomerulus and its adherent Af-Art were microdissected under a stereomicroscope (SMZ1500, Nikon, Yuko, Japan). The microdissected sample was transferred to a temperature-regulated chamber mounted on an inverted microscope (Axiovert 100TV, ZEISS, NY, USA) together with DMEM. The glomerulus was held with micropipette and Af-Art was cannulated and perfused with a set of micropipettes. The intraluminal pressure of the perfused Af-Art was maintained at 60 mmHg throughout the experiment. The chamber was perfused with DMEM with or without losartan (10⁻⁶mol/L) at 1-1.5 mL/min
at 37°C. After 30 minutes of an equilibration period, the dose response curves of Ang II (10^{-12} to 10^{-6} mol/L in DMEM, in random order) with or without losartan (10^{-6} mol/L) were obtained. Each concentration of Ang II was perfused for 5 minutes, and the maximum constrictive response of the Af-Art was measured; the bath was then switched to DMEM for 15 minutes before next dose of Ang II stimulation.

**Real-time PCR.** The same method was used to isolate the Af-Art as we reported previously (Lu *et al.* 2015, Lu *et al.* 2016, Zhang *et al.* 2014). Af-Arts were isolated from kidney slices of the mice without Ang II infusion in ice-cold DMEM under a stereomicroscope. A single Af-Art was transferred into RLT buffer (RNeasy Mini Kit; Qiagen, Venlo, Netherlands) for RNA extraction. To avoid RNA degradation, the time for dissection was limited to 30 minutes after kidney removal.

Total RNAs (20-30 ng/μl) were extracted from an Af-Art using RNase Mini Kit according to the manufacturer's instructions. After digestion with RNase-free DNase (Promega, WI, USA) to eliminate the genomic contamination, the cDNAs were synthesized with a reverse transcription system using oligo d(T) primer and used as templates. Quantitative PCR analysis was performed using iQ SYBR Green Supermix (Bio-Rad, CA, USA) and CFX96 Real-Time Detection System (Bio-Rad, CA, USA) according to the manufacturer's protocol. Reaction conditions for AT1 and AT2 receptors were 95 °C for 1 min, followed by 40 cycles of 95 °C for 15 s, then 60 °C for 30 s; and for Mas receptor were 95 °C for 3 min, 1 cycle; 95 °C for 10 s, 56 °C for 30 s, 50 cycles; 72 °C for 10 s, 1 cycle. The reaction of each sample was performed in triplicate. Dissociation analysis was performed at the end of each PCR reaction to confirm the amplification specificity. After the PCR program, data were analyzed and quantified with the
comparative $C_t$ method ($2^{-\Delta\Delta C_t}$) based on $C_t$ values to calculate the relative mRNA expression level. The primer sequences and accession numbers are listed in Table 1. The expected size of PCR products of AT1, AT2, and Mas was 240 bp, 230 bp and 175 bp respectively (Prasad et al. 2014, Wang et al. 2017).

Table 1. The mouse primers sequences and accession number.

<table>
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<tr>
<th>Gene name</th>
<th>Sequences</th>
<th>Accession number</th>
</tr>
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<tbody>
<tr>
<td>AT1</td>
<td>Forward: 5'-ATCGCTACCTGGCCATTGTC-3' Reverse: 5'-GGAAGCCCAGGATGTTCTTG-3'</td>
<td>NM_177322.3</td>
</tr>
<tr>
<td>AT2</td>
<td>Forward: 5'-TTACCAGCAGCCGTCCTTTT-3' Reverse: 5'-GTCAGCCAAGGCCAGATTGA-3'</td>
<td>NM_007429.5</td>
</tr>
<tr>
<td>Mas</td>
<td>Forward: 5'-AGGGTGACTGACTGAGTTGG-3' Reverse: 5'-GAAGGTAAGAGGACAGGAGGC-3'</td>
<td>NM_008552</td>
</tr>
<tr>
<td>β-Actin</td>
<td>Forward: 5'-GTCCTCACCCTCCCAAAG-3' Reverse: 5'-GCTGCTCAACCATCCTCAACC-3'</td>
<td>NM_007393.3</td>
</tr>
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**Statistics.** The number of mice in each experiment was determined by power analysis based on $p$-value=0.05 and a power of 80% (Charan & Kantharia 2013, Keir et al. 2017). Data were presented as mean ± SEM. A Student’s t-test was used to determine statistical differences. An ANOVA with post hoc test was used for within-group and between-group measurements. A two-way ANOVA was used to compare dose response curves in isolated arterioles. The difference was considered to be significant for a $p$-value<0.05.

**Results**

MAP and renal hemodynamic responses to an acute Ang II injection. To determine whether the diabetic mice are more sensitive to acute Ang II stimulation, MAP and RBF were measured and compared between anesthetized non-diabetic and diabetic
mice (Figure 20A). Baseline MAP was 82.9±1.1 and 83.7±3.1 mmHg in diabetic and non-diabetic mice, respectively. MAP increased by 43.5±2.6% to 119.1±3.8 mmHg in diabetic mice following acute Ang II injection (p<0.01 vs. baseline). However, MAP only increased by 26.8±2.3% to 106.2±3.5 mmHg in non-diabetic mice (Figure 20B and 20C) (p<0.01 vs. baseline). Baseline RBF was significantly higher in diabetic mice (1.53±0.18 mL/min) compared with non-diabetic mice (0.98±0.12 mL/min) (p<0.05 vs. non-diabetes). RBF decreased by 83.4±5.4% to 0.25±0.07 mL/min in diabetic mice (p<0.01 vs. baseline) but only decreased by 47.1±13.7% to 0.52±0.14 mL/min in non-diabetic mice (p<0.01 vs. baseline) following an acute Ang II injection (Figure 20D and 20E). Baseline RVR was significantly lower in diabetic mice compared with non-diabetic mice (Figure 20F) (p<0.05 vs. non-diabetes). RVR increased about 8 folds in diabetic mice (p<0.05 vs. baseline) and just increased about 1.5 folds in non-diabetic mice (Figure 20G) (p<0.05 vs. baseline). There was no functional response to an injection of 20 μl saline.
Representative image (A) demonstrates the change of MAP and RBF after acute Ang II injection. Acute Ang II injection induced a greater increase in MAP (B and C; *p<0.01 vs baseline, #p<0.05 vs non-diabetes) and a greater decrease in RBF (D and E; *p<0.01 vs baseline, #p<0.05 vs non-diabetes) in diabetic mice compared with non-diabetic mice. Calculated RVR was also greater in diabetic mice than in non-diabetic mice following Ang II infusion (F and G; *p<0.05 vs. baseline, #p<0.05 vs. non-diabetes). (Diabetes: n = 10, non-diabetes: n = 7).

Figure 20. Responses of MAP, RBF and RVR to acute Ang II infusion.
**MAP response to chronic Ang II infusion.** To determine whether the sensitivity of blood pressure to chronic Ang II infusion is enhanced in diabetes, we measured MAP with telemetry in diabetic mice infused with a slow pressor dose of Ang II and compared with non-diabetic mice (Figure 21A). Baseline MAP was 101.6±3.4 mmHg in diabetic mice and 98.4±2.2 mmHg in non-diabetic mice. MAP increased by 54.9±6.1% to 157.4±4.6 mmHg in diabetic mice following 4-week Ang II infusion (p<0.01 vs. baseline). However, MAP only increased by 32.8±4.4% to 130.7±3.6 mmHg in non-diabetic mice (Figure 21B and 21C) (p<0.01 vs. baseline). There were no significant differences in heart rate between diabetes and non-diabetes (Figure 21D).

*Figure 21. Changes in MAP in response to chronic Ang II infusion.*

Chronic Ang II infusion caused a greater increase in MAP in diabetic mice compared with non-diabetic mice. (n=6) *p<0.01 vs baseline; *p<0.01 vs non-diabetes.
**Diabetes enhances the constrictive effect of Ang II on Af-Art.** To determine whether Ang II-induced constriction of the Af-Art is enhanced in diabetes, the dose-response curves for Ang II (10^{-12} to 10^{-6} mol/L) in isolated perfused Af-Art was measured in diabetic mice and compared with non-diabetic mice (Figure 22A). In diabetic mice, the basal diameter of the Af-Art was 12.91±0.34 μm. Ang II (10^{-12} to 10^{-6} mol/L) reduced the Af-Art diameter in a dose-dependent manner. The diameter was reduced to 56.3±3.2% of baseline at 10^{-9} mol/L Ang II (p<0.01 vs. baseline) and 34.1±4.3% of baseline at 10^{-6} mol/L Ang II (p<0.01 vs. baseline). In non-diabetic mice, the Af-Art diameter was 11.76±0.98 μm at baseline and reduced to 83.1±6.6% of baseline at 10^{-9} mol/L of Ang II (p<0.05 vs. baseline) and to 52.1±5.3% of baseline at 10^{-6} mol/L of Ang II (p<0.01 vs. baseline). Ang II-induced contraction was significantly enhanced in diabetic mice (Figure 22B and 22C) (p<0.05 vs. non-diabetes).
Representative images (A) demonstrated Af-Art constriction induced by Ang II in non-diabetic and diabetic mice. Ang II-induced contraction was significantly enhanced in diabetic mice (B and C; *p<0.05 vs. non-diabetes). (Diabetes: n=11, non-diabetes: n=8).

Figure 22. Dose-response curve of the afferent arteriole (Af-Art) to Ang II.
Losartan blocked Ang II induced vasoconstriction. To determine whether Ang II constricts Af-Art via activation of AT1 receptors, the dose response curve for Ang II (10^{-12} to 10^{-6} mol/L) plus losartan (10^{-6} mol/L) was measured and compared between diabetic and non-diabetic mice. Ang II (10^{-12} to 10^{-6} mol/L) did not reduce the Af-Art diameter in the presence of losartan both in diabetic and non-diabetic mice (Figure 23A and 23B).

![Graph A](image1.jpg)

**Figure 23. Effect of AT1 inhibition on Ang II-induced vasoconstriction of the Af-Art.**

AT1 receptor antagonist losartan blocked Ang II-induced (10^{-12} to 10^{-6} mol/L) vasoconstriction of the Af-Art in both diabetic and non-diabetic mice. (Diabetes: n=7, non-diabetes: n=6)

**AT1 receptor mRNA levels.** To determine whether diabetes alters Ang II receptor expression levels, we dissected Af-Art from non-diabetic and diabetic mice and measured...
the expression of AT1 and AT2 receptors mRNA by real-time PCR. The AT1 receptor mRNA level in the Af-Art was 9.4±0.5 times higher in diabetic mice than that in non-diabetic mice (p<0.01 vs. non-diabetes) (Figure 24A). The AT2 receptor mRNA level was similar in diabetic and non-diabetic mice (Figure 24B).

![Figure 24. mRNA levels of AT1 and AT2 receptors in the Af-Art.](image)

AT1 receptor mRNA level was significantly higher in diabetic mice than that in non-diabetic mice in the isolated Af-Arts (A; *p<0.01 vs. non-diabetes). AT2 receptor mRNA level in diabetic mice was similar as that in non-diabetic mice in the Af-Arts (B). (Diabetes: n=10, non-diabetes: n=10)

**Discussion**

In the present study, we demonstrated that the responses of MAP and RBF to both acute and chronic Ang II infusion were enhanced in alloxan induced diabetic C57BL/6
mice. The constrictive response of the Af-Art to Ang II was exaggerated in diabetic mice associated with upregulated expression of AT1 receptor. AT1 receptor antagonist losartan blocked Ang II induced vasoconstriction in both non-diabetic and diabetic mice.

RAS inhibitors including angiotensin converting enzyme inhibitors and angiotensin receptor blockers have been recommended as first-line anti-hypertensive medications for diabetic patients. RAS inhibition showed a distinctive benefit on cardiovascular outcomes for diabetic patients with hypertension, compared with the other anti-hypertensive agents like beta blockers and diuretics (2002, Psaty et al. 1997), suggesting an important role of the RAS in the development of hypertension in DM (2000, Patel et al. 2007). In the present study, we compared MAP in diabetic and non-diabetic mice in response to either bolus intravenous injection or chronic infusion of Ang II. The higher MAP in the diabetic group indicates that the responsiveness of blood pressure to Ang II is exaggerated in diabetic mice. This is consistent with the previous clinical studies where the response of blood pressure to acute Ang II infusion was more sensitive in diabetic patients compared with non-diabetic subjects (Beretta-Piccoli & Weidmann 1981, Drury et al. 1984, Weidmann et al. 1985).

A previous study with kidney cross-transplantation between wild-type and AT1 receptor-deficient mice demonstrated the predominant role of the renal AT1 receptor in the development of Ang II-dependent hypertension. The mice with kidney-specific AT1 receptor elimination via transplantation were relatively resistant in hypertensive response to Ang II infusion (Crowley et al. 2006). In non-diabetic animals, both acute and chronic Ang II infusion intensify blood pressure along with a marked increase in RVR (Fagard et al. 1976, Mattar et al. 1996, Venegas-Pont et al. 2011). In the present study, we compared
RBF and calculated RVR between diabetic and non-diabetic mice in response to bolus intravenous injection of Ang II. We found that the renal hemodynamic response to acute Ang II infusion was greater in diabetic mice compared with non-diabetic mice.

As the major resistant vessels in the kidney, Af-Arts account for more than 50% of the RVR (Arima & Ito 2003, Burke et al. 2014, Carlstrom et al. 2015). To investigate the vascular response to Ang II in DM, the Ang II dose-response curves of the Af-Arts from diabetic and non-diabetic mice were compared. We found that the vasoconstriction of renal Af-Art in response to Ang II was significantly exaggerated in diabetic mice. Consistent with our finding, enhancement in Ang II dependent vasoconstriction has been found in several different kinds of vessels including mesenteric bed, carotid artery and renal artery from diabetic rats, indicating that increased responsiveness to Ang II might be a general characteristic of the universal vasculatures in diabetes (Benter et al. 2007).

Effects of Ang II on Af-Arts are primarily mediated by two types of Ang II receptors, AT1 and AT2 receptors (Guo et al. 2001, Higuchi et al. 2007, Kaschina & Unger 2003). Activation of the AT1 receptor in vascular smooth muscle cells stimulates Ca\(^{2+}\) influx and subsequently induces vasoconstriction (Kanaide et al. 2003, Loutzenhisser & Loutzenhisser 2000). On the contrary, AT2 receptor mediates the endothelium-dependent vasodilatation via stimulation of nitric oxide and bradykinin (Arima et al. 1997, Siragy et al. 1999, Siragy & Carey 1997). The renal expression of angiotensin receptors in diabetes has been investigated by several laboratories. The renal AT1 receptor protein level in diabetic rats was reported to be higher than in non-diabetic rats (Harrison-Bernard et al. 2002). However, several other studies reported that the kidney AT1 receptor was downregulated in diabetic animal models and diabetic patients (Burns 2000, Mogyorosi & Sonkodi 1999,
Also, a lower expression level of the AT1 receptor was also found in hypertensive animals with diabetes (Bonnet et al. 2002, Burns 2000, Zimpelmann et al. 2000). In addition, Wagner et al. demonstrated that the mRNA level of the AT1 receptor in human whole kidney biopsy samples was significantly lower in patients with Type II DM than non-diabetics (Wagner et al. 1999). The reasons for the inconsistent observations in AT1 receptor expression levels in diabetes are not clear, but might be due to the different regions of the kidney that were examined. Regarding the AT2 receptor, a downregulation of expression in the kidney was demonstrated in both early streptozotocin-induced diabetic Sprague-Dawley rats (Wehbi et al. 2001) and spontaneously hypertensive rats with long-term diabetes (Bonnet et al. 2002). Moreover, the localization of Ang II receptors is widely distributed in the kidney. Both immunohistochemical and Ang II binding studies demonstrated that AT1 receptor was abundant in the cortical vasculature and S3 segment of the proximal tubules in the outer medulla, with lesser expression in the T and collecting ducts (Meister et al. 1993, Miyata et al. 1999, Mujais et al. 1986). The major distribution of AT2 receptor in the kidney was indicated in interlobular arteries, afferent arterioles, glomeruli, proximal tubules and collecting ducts (Matsubara et al. 1998, Siragy 2010, Wehbi et al. 2001). Thus, the expression levels of Ang II receptors in the kidney homogenate may not reflect their specific level in Af-Art. To determine the expression of Ang II receptors exclusively in Af-Art, we isolated Af-Art and then measured the mRNA level of AT1 and AT2 receptors. We found that AT1 receptor expression was over 10-fold higher in diabetic mice than non-diabetic mice, whereas the level of AT2 receptor was similar. This finding is consistent with the study by Sodhi et al. that the medium with high glucose upregulated AT1 receptor
expression in cultured vascular smooth muscle cells (Sodhi et al. 2003). However, our results are not inconsistent with the previous report that both AT1-A and AT1-B receptor protein levels in renal arterioles, measured by immunohistochemistry were lower in STZ induced diabetic Wistar rats (Hall 1986, Razga et al. 2014).

Taken together, these findings indicate that exaggerated Af-Art vasoconstrictive response to Ang II, mediated by the increased expression of AT1 receptor, which may contribute to the enhanced blood pressure and renal hemodynamic responses to Ang II in DM. Therefore, the current study reveals a mechanism that may contribute to the development of hypertension in DM.

Diabetic subjects have a higher prevalence of hypertension, but the mechanisms have not been fully elucidated. The findings of the present study provide a potential mechanism for the high prevalence of hypertension in diabetic patients. In the present study, we found that blood pressure and renal hemodynamic responses to Ang II are enhanced in alloxan induced type I diabetic mice. Furthermore, we demonstrated that DM exaggerates the constrictive responsiveness of the Af-Art to Ang II, which was mediated by the upregulation in the expression of AT1 receptor since the effect of Ang II was blocked by AT1 receptor antagonist losartan. Plasma renin-angiotensin II level is suppressed in early diabetes (Amemiya et al. 1990, Kennefick & Anderson 1997), which permits low vascular tone of the Af-Art and glomerular hyperfiltration in early diabetes. On the other hand, mild or moderate elevation of circulating or local renin-Ang II that may not be able to increase blood pressure in non-diabetic patients could induce hypertension in diabetes.
Project III: enhanced expression and activity of Nox2 and Nox4 in the macula densa in Ang II-induced hypertensive mice

Abstract

Nox2 and Nox4 are the isoforms of NAD(P)H oxidase (Nox) expressed in the macula densa (MD). MD-derived superoxide (O$_2^-$), primarily generated by the Nox2, is enhanced by acute Ang II stimulation. However, the effects of chronic elevations in Ang II during Ang II-induced hypertension on MD derived O$_2^-$ are unknown.

We infused a slow-pressor dose of Ang II (600 ng/min/kg) for two weeks in C57BL/6 mice and found mean arterial pressure (MAP) was elevated by 22.3±3.4 mmHg (p<0.01). We measured O$_2^-$ generation in isolated and perfused MD and found O$_2^-$ generation by the MD was increased from 9.4 ± 0.9 unit/min in controls to 34.7 ± 1.8 unit/min in Ang II-induced hypertensive mice (p < 0.01). We stimulated MMDD1 cells, a MD like cell line, with Ang II and found O$_2^-$ generation increased from 921 ± 91 to 3687 ± 183 unit/min/10$^5$ cells, which was inhibited with apocynin, oxypurinol, or NS-398 by 46, 14, and 12% respectively. We isolate the MD cells using laser capture microdissection and measured mRNA levels of Nox. Nox2 and Nox4 levels increased by 3.7±0.17 and 2.6±0.15 folds in the Ang II-infused mice compared to controls. In the MMDD1 cells treated with Nox2 or Nox4 siRNAs, Ang II-stimulated O$_2^-$ generation was blunted by 50% and 41%, respectively. In the cells treated with p22phox siRNAs, Ang II-stimulated O$_2^-$ generation was completely blocked.

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In conclusion, we found that subpressor dose of Ang II enhances \( \text{O}_2^- \) generation in the MD and the sources of this \( \text{O}_2^- \) are primarily the Nox2 and Nox4.

**Introduction**

Kidneys are vital in maintaining adequate extracellular fluid volume, and they do so by regulating the excretion of sodium and water. Abnormalities in sodium and water excretion may result in inappropriate salt and water retention, which can facilitate hypertension. One of the mechanisms that the kidney possesses to regulate salt excretion is via the macula densa (MD). The MD cells are modified epithelial cells located at the distal segment of the TAL which comprise an integral component of the juxtaglomerular apparatus. The MD cells serve as luminal sensors of sodium chloride (NaCl) concentration; it senses increases in NaCl delivery to which it responds by initiating a sequence of events which results in an increase in adenosine and or ATP release. These in turn constrict the afferent arterioles, lower capillary hydraulic pressure and decrease SNGFR, and decreases tubular flow back to normal levels. These series of events are collectively termed as the TGF response. The TGF response establishes a negative feedback by which a change in tubular flow to the MD induces a reciprocal change in SNGFR, thus preventing acute fluctuations of flow and NaCl delivery in the distal nephron (Briggs & Schnermann 1986, Ollerstam *et al.* 1997, Ren *et al.* 2000b). Hence, the TGF limits urinary volume and sodium excretion, and thus may contribute to maintain salt and water homeostasis. Any abnormality in the TGF response may alter salt and water balance. Indeed, several studies have reported that TGF responses are enhanced in several experimental models of hypertension, and that this may be contributing to the hypertension (*Aviv et al.* 2004, Brannstrom *et al.* 1996, Huang *et al.* 1988, Kotchen *et al.*
The MD cells not only initiate the TGF response, but the same signals also regulate renin release (Schnermann & Levine 2003), which also plays a key role in regulating renal hemodynamics, salt and water excretion, and blood pressure. Due to the importance of these two mechanisms, they are tightly regulated by various factors including angiotensin II (Ang II), nitric oxide (NO) and superoxide (O$_2^-$) (Wang et al. 2001). Consequently, interactions between these factors may have an important effect on renal function. For example, NO in the MD blunts TGF response via a cGMP dependent pathway. O$_2^-$ enhances TGF responsiveness mediated by scavenging NO and reducing NO bioavailability in the MD (Liu et al. 2002b, Liu et al. 2004b, Ren et al. 2002a, Zhang et al. 2009). Thus, any factor which alters the levels of this O$_2^-$ may potentially modulate TGF resulting in abnormal salt and water homeostasis. Therefore, it is important to understand the sources and mechanisms that alter O$_2^-$ production in the MD.

The main source of O$_2^-$ in the kidney is nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidase (Nox), which has been shown to be present in the renal cortex, medulla and blood vessels (Gill & Wilcox 2006). This O$_2^-$ has been implicated in several models of hypertension. Our recent studies have demonstrated that the Nox2 and Nox4 are the primary isoforms of Nox expressed in the MD (Zhang et al. 2009). Moreover, we found that MD-derived O$_2^-$ is enhanced by acute Ang II stimulation and by increases in NaCl, and that this increase in O$_2^-$ is primarily generated by the Nox2 isoform (Fu et al. 2010, Zhang et al. 2009). However, the effects of chronic elevations in Ang II on MD derived O$_2^-$ are unknown. Because inappropriate chronic elevations in Ang II may cause
enhanced TGF and thus salt-sensitive hypertension, in this study we determined the changes in expression and function of Nox in the MD following chronic Ang II stimulation.

Methods

All procedures and experiments were approved by the Institutional Animal Care and Use Committee at the University of Mississippi Medical Center. All chemicals were purchased from Sigma (St. Louis, MO) except as indicated.

Telemetry transmitter implantation: Briefly, C57BL/6 mice (18-20 g, Harlan) were anesthetized with inhaled isoflurane (Butler chemicals, UK). A small incision was made in the middle of neck for insertion of the telemetry transmitter (PA-C10, Data Sciences International). The catheter of the transmitter was placed in left carotid artery and advanced down to the aortic arch. The body of the transmitter was placed subcutaneously in the right ventral flank of the animal. The mice were allowed to recover for ten days.

Minipump insertion: Osmotic minipumps (Alzet Corp) were primed and filled with either Ang II (600ng/kg/min) or saline (control). On the day of surgery, the animals which had ten days to recover from the telemetry transmitter implantation were again anesthetized with isoflurane. Under sterile conditions, a small incision was made in the mid scapular area on the animal’s back. Using a hemostat, the subcutaneous tissue was separated and spread so as to create a small pouch in which the minipump was inserted. Following insertion, the wound was sutured and the animals were allowed to recover for a day. Measurements of the mean arterial pressure (MAP) were started on the second day after minipump implantation at ten minute intervals.
Laser capture micro dissection (LCM) and real-time PCR: Laser capture microdissection (LCM) and real-time PCR were used to isolate MD cells and to measure Nox2 and Nox4 mRNA levels with methods we have described previously (Fu et al. 2012, Lu et al. 2010, Zhang et al. 2009). Briefly, kidneys from C57BL/6 mice were removed and snap-frozen in Optimal Cutting Temperature Medium. Eight-micrometer-thick frozen sections were obtained, and then stained and dehydrated using an Arcturus Histogene frozen section staining kit (Life Technologies, Carlsbad, CA). The MD cells were dissected with an Arcturus Laser Capture Microdissection System (Model Veritas). Real-time PCR was used to quantify mRNA levels of the Nox2 and Nox4. Total RNA from the MMDD1 cells was extracted with an RNeasy Micro kit (Qiagen), following the manufacturer’s instructions. One µg of total RNA was reverse transcribed at 25°C for 5min, 42°C for 45min, 85°C for 5min using an iScript cDNA Synthesis Kit (Bio-Rad, USA) following the manufacturer’s instructions. Real-time PCR was performed in a C1000TM Thermal Cycler real-time PCR machine (Bio-Rad). The β-actin was used as a housekeeping gene.

Isolation and micro-perfusion of Juxtaglomerular apparatus (JGA) and measurement of O2− with dihydroethidium: We used methods similar to those we described previously to isolate and micro-perfuse the MD with attached glomerulus (Fu et al. 2012, Liu et al. 2004b). Briefly, the kidneys were removed and sliced along the cortico-medullary axis. Single superficial intact glomeruli were microdissected together with adherent tubular segments consisting of portions of the TAL, macula densa and early distal tubule. Using a micropipette, samples were transferred to a temperature-regulated chamber mounted on an inverted microscope (TE2000-S, Nikon). The glomeruli were
positioned so the macula densa could be visualized using a holding pipette. TALs were cannulated with an array of glass pipettes. The MD’s were perfused with physiological saline containing (in mmol/L) 10 HEPES, 1.0 CaCO₃, 0.5 K₂HPO₄, 4.0 KHCO₃, 1.2 MgSO₄, 5.5 glucose, 0.5 Na acetate, 0.5 Na lactate, and pH 7.4, NaCl was 10 or 80 mmol/L. Luminal perfusion rate was 40 nL/min and controlled with a pump (11 plus, Harvard Apparatus, Holliston, MS). The bath consisted of Modified Essential Medium containing 0.15% bovine serum albumin and was exchanged continuously at a rate of 1 mL/min.

We used a O₂⁻ sensitive fluorescent dye, dihydroethidium, to detect O₂⁻ levels as we recently described (Liu et al. 2008). Briefly, once the TAL was perfused, MD cells were loaded with 10 µM dihydroethidium in 0.1% dimethyl sulfoxide (DMSO) plus 0.1% pluronic acid from the lumen for 30 min then washed for 20 min. The loaded MD cells were exposed to 380 and 490 nm light to excite dihydroethidium and oxyethidium, respectively. Emitted fluorescence from dihydroethidium was recorded using a 420 nm dichroic mirror with a 460/50 nm band-pass filter; for oxyethidium we used a 565 nm dichroic mirror with a 605/55 nm band-pass filter. Square-shaped regions of interest (ROIs) were set inside the cytoplasm of MD cells and their mean intensity recorded every 5 sec for 2 min. We recorded and calculated the rate of the changes for oxyethidium/dihydroxyethidium as an indicator of O₂⁻ production. Since we previously found that increased luminal NaCl induced NO release by the MD, we added the NOS inhibitor N-nitro-L-arginine methyl ester (L-NAME) (10⁻⁴ mol/L) to the bath and lumen while measuring O₂⁻ to eliminate its reaction with NO as we reported previously (Liu et al. 2007, Liu et al. 2008).
Measurement of $\text{O}_2^-$ with lucigenin-enhanced chemiluminescence in MMDD1 cells: Our experiments utilized MMDD1 cells, a MD-like cell line (kindly provided by Dr. J. Schnermann, National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD) in a manner similar to our previously described studies (Fu et al. 2010, Zhang et al. 2009, Zhu et al. 2011). MMDD1 cells were stimulated by Ang II (10^{-6}–10^{-10} mol/L) for 12 hrs. $\text{O}_2^-$ production in the MMDD1 cells was determined using a lucigenin-enhanced chemiluminescence assay, as described previously (Fu et al. 2010, Zhang et al. 2009). Briefly, MMDD1 cells (10cm-dish) were washed with PBS twice, trypsinized in the dish and kept in 9 mL or 12mL Krebs/Hepes buffer (containing in mmol/L: 115 NaCl, 20 HEPES, 1.17 K$_2$HPO$_4$, 1.17 MgSO$_4$, 4.3 KCl, 1.3 CaCl$_2$, 25 NaHCO$_3$, 11.7 glucose, 0.1 NAD(P)H, with pH adjusted to 7.4). Then lucigenin (5×10^{-6}mol/L) was added to the samples which were incubated for 30 min at 37 °C with oxygen bubbling. A 0.5 ml sample was transferred into a 1.6-mL polypropylene 8×50 mm tubes, and then using a Sirius luminometer (Berthold Detection Systems, Pforzheim, Germany), $\text{O}_2^-$ was measured following the manufacturer’s instructions. Luminescence was measured for 10 sec with a delay of 5 sec.

Preparations for small interfering RNA (siRNA): Nox2, Nox4 and p22phox siRNAs were designed and synthesized by Santa Cruz Biotechnology (Santa Cruz, CA). siRNA transfection was performed following the manufacturer’s instructions as described earlier (Zhang et al. 2009, Zhu et al. 2011). Briefly, the transfection of the siRNA was performed using TransMessenger Transfection Reagent from QIANGEN (Germantown, MD) according to the manufacturer’s instructions. Scrambled siRNA were synthesized and used as negative controls. Twenty four hours before transfection, MMDD1 were
transferred onto 6-well plates and transfected with 2 µg of each siRNA duplex using TransMessenger transfection reagent for 3 hours in medium devoid of serum and antibiotics. The MMDD1 cells were then washed once with PBS and grown in complete medium. Gene silencing was monitored by measuring RNA after incubation for 24 hours.

**Statistical analysis:** Data were collected as repeated measures over time under different conditions. We tested only the effects of interest, using analysis of variance (ANOVA) for repeated measures and a post-hoc Fisher LSD test or a Student’s paired t-test when appropriate. The changes were considered to be significant if $p < 0.05$. Data are presented as mean ± SEM.

**Results**

**Subpressor Ang II infusion induced hypertension in mice:** To demonstrate the effect of a slow-pressor dose of Ang II on blood pressure, we infused Ang II with a minipump at a dose of 600 ng/min/kg for two weeks and measured MAP with telemetry in C57BL/6 mice. We found the basal MAP was 101±3.1 mmHg and this starts to gradually elevate from day 5 of minipump insertion in the angiotensin group. The blood pressure continued to rise and reached a peak of 123±3.8 mmHg on days 10-14 of the infusion. ($P < 0.01$; n= 7). The MAP of control mice infused with saline did not vary significantly (n=5, Figure 25).
A slow pressor dose of Ang II at 600 ng/min/kg was infused for 2 weeks in C57BL/6 mice. Mean arterial pressure was elevated to about 22.3±3.4 mmHg (* p <0.01 vs. basal, n = 7). While the blood pressure was no significant change in mice infused with saline (n=5).

In isolated and perfused the JGA, O$_2^-$ generation by the MD was 9.4 ± 0.9 unit/min in mice infused with saline. It increased to 34.7 ± 1.8 unit/min in mice infused with Ang II for 2 weeks with minipumps (n = 6). In the presence of apocynin, an Nox inhibitor, O$_2^-$ generation by the MD was 2.1 ± 0.3 unit/min in controls and increased to 12.4 ± 2.3 unit/min in Ang II-induced hypertensive mice (# p < 0.01, * p<0.05 vs. without apocynin, n = 5).
**O$_2^-$ generation by the MD is enhanced in Ang II-induced hypertensive mice:**

To determine whether the O$_2^-$ generation by the MD cells increases following Ang II infusion, we isolated and perfused the JGA and measured O$_2^-$ generation using dihydroethidium in mice infused with Ang II or saline for 2 weeks with minipumps. The MD was perfused with an 80 mM NaCl solution in the presence of L-NAME (10^{-4} mol/L) in the bath and lumen. O$_2^-$ generation by the MD was 9.4 ± 0.9 unit/min in controls. It increased to 34.7 ± 1.8 unit/min in Ang II-induced hypertensive mice (p < 0.01, n =6, Figure 26).

To determine the source of O$_2^-$ generation, we used the Nox inhibitor apocynin and repeated the above protocol. In the presence of apocynin, O$_2^-$ generation by the MD was 2.1 ± 0.3 unit/min in controls and increased to 12.4 ± 2.3 unit/min in Ang II-induced hypertensive mice (p < 0.01, n = 5, Figure 26). These data indicate that there is a significant increase in the MD O$_2^-$ generation in Ang II-hypertensive mice and that Nox is an important source of this O$_2^-$.

**Ang II increased O$_2^-$ generation in cultured MMDD1 cells:** To further study the source of Ang II-induced O$_2^-$ generation in the MD, we stimulated the cultured MMDD1 cells with Ang II and measured O$_2^-$ generation. First, we tested the dose and time response curve. As shown in Figure 27A, when the MMDD1 cells were stimulated with different concentration of Ang II for 12 hours, the Ang II at a concentration of 10^{-8} mol/L was the lowest concentration with prominent effect on O$_2^-$ generation. Then we tested the time response curve using 10^{-8} mol/L Ang II and found that 12 hours of stimulation induced substantial O$_2^-$ generation (Figure 27B). Therefore, in the following experiments, we stimulated the MMDD1 cells with 10^{-8} mol/L Ang II treatment for 12 hours.
Figure 27. Dose and time response curve of Ang II-induced $O_2^-$ generation in cultured MMDD1 cells.

A. MMDD1 cells were stimulated with different concentration of Ang II for 12 hours. The Ang II at a concentration of $10^{-8}$ and $10^{-7}$ mol/L was most effective on $O_2^-$ generation (* $p<0.01$ vs. $10^{-10}$ mol/L, n = 41). B. $O_2^-$ generation in MMDD1 cells was correspondingly increase in response to a $10^{-8}$ mol/L Ang II stimulation for different time and reached a plateau at 12 hours (* <0.01 vs. basal, n = 37).

Under basal conditions, $O_2^-$ generation was $921 \pm 91$ unit/min/10$^5$ cells. It increased to $3687 \pm 183$ unit/min/10$^5$ cells. In the presence of a Nox inhibitor apocynin (10$^{-5}$ mol/L), xanthine oxidase inhibitor oxypurinol (10$^{-4}$ mol/L), or COX-2 inhibitor NS-398 (10$^{-6}$ mol/L), the Ang II-stimulated $O_2^-$ generation was $1963 \pm 122$, $3189 \pm 83$, and $3246$
± 96 unit/min/10^5 cells, respectively (Figure 28). These data again point to the fact that
Ang II stimulated O_2^- generation in the MMDD1 cells arises primarily from Nox.

![Bar graph showing effect of different antagonists on Ang II-induced O_2^- generation in MMDD1 cells.]

Figure 28. Effect of different antagonists on Ang II-induced O_2^- generation in MMDD1 cells.

Ang II stimulation increased O_2^- generation from 921 ± 91 to 3687 ± 183 unit/min/10^5 cells (* p<0.01 vs. control). In the presence of a Nox inhibitor apocynin (10^-5 mol/L), xanthine oxidase inhibitor oxypurinol (10^-4 mol/L), or COX-2 inhibitor NS-398(10^-6 mol/L), the Ang II-stimulated O_2^- generation was 1963 ± 122, 3189 ± 83, and 3246 ± 96 unit/min/10^5 cells, respectively (** p<0.05 vs. Ang II; # p<0.01 vs. Oxypurinol and NS-398, n = 29).

Nox2 and Nox4 mRNA levels in the MD are increased in Ang II-induced hypertension: To determine the changes of Nox expression in the MD induced by Ang II, laser capture microdissection technique was utilized to isolate the MD cells in mice infused with either subpressor Ang II or saline. Next, the Nox2 and Nox4 levels were measured with real-time PCR. Nox2 mRNA levels in the MD increased 3.7±0.17 folds in the Ang II-infused mice compared to controls (p < 0.01, n = 5). Nox4 mRNA levels in the MD increased 2.6±0.15 folds in the Ang II-infused mice compared to controls (p < 0.01, n = 5, Figure 29). These data indicated that, both Nox2 and Nox4 isoforms of Nox in the MD significantly increased in subpressor Ang II-induced hypertension.
Nox2 mRNA levels in the MD isolated with laser capture microdissection increased 3.7±0.17 folds in the Ang II-infused mice compared to controls (* p < 0.01 vs. saline, n = 5). Nox4 mRNA levels in the MD increased 2.6±0.15 folds in the Ang II-infused mice compared to controls (* p < 0.01 vs. saline, n = 5).

Ang II generated O$_2^-$ in the MD originates from both Nox2 and Nox4 isoforms of Nox in the MMDD1 cells: To determine which isoform/s of Nox is/are the primary source of Ang II-stimulated O$_2^-$ generation, we used Nox2 and Nox4 siRNAs to silence the Nox2 and Nox4 genes and used p22phox siRNAs to knock-down the function of both Nox2 and Nox4. Ang II stimulation increased O$_2^-$ generation in the MMDD1 cells treated with scrambled siRNA from 967 ± 42 to 3278 ± 94 unit/min/10$^5$ cells. In the MMDD1 cells treated with Nox2 or Nox4 siRNAs, Ang II-stimulated O$_2^-$ generation was 1632 ± 61 and 1932 ± 57 unit/min/10$^5$ cells, respectively. In the cells treated with p22phox siRNAs, Ang II-stimulated O$_2^-$ generation was blocked (784 ± 83 unit/min/10$^5$ cells, Figure 30A). To further confirm the contributions from NOX2 and NOX4, we treated the MMDD1 cells with both NOX2 and NOX4 siRNAs. Ang II stimulation increased O$_2^-$ generation in the MMDD1 cells treated with scrambled siRNAs from 853 ± 51 to 2841 ± 83 unit/min/10$^5$ cells. In the
MMDD1 cells treated with Nox2 and Nox4 siRNAs, Ang II-stimulated O$_2^-$ generation was 718 ± 44 unit/min/10$^5$ cells (Figure 30B). These data indicate that Ang II enhances O$_2^-$ generation from both Nox2 and Nox4 isoforms of Nox in the MMDD1 cells.

![Graph](image)

**Figure 30. Ang II generated O$_2^-$ in the MD originates from both Nox2 and Nox4 isoforms of Nox in the MD.**

**A.** Ang II stimulation increased O$_2^-$ generation in the MMDD1 cells treated with scrambled siRNA from 967 ± 42 to 3278 ± 94 unit/min/10$^5$ cells (* p<0.01 vs. control, n = 54). In the MMDD1 cells treated with Nox2 or Nox4 siRNAs, Ang II-stimulated O$_2^-$ generation was reduced to 1632 ± 61 and 1932 ± 57 unit/min/10$^5$ cells, respectively (#p<0.01 vs. Ang II, n = 23). In the cells treated with p22phox siRNAs, Ang II-stimulated O$_2^-$ generation was blocked (784 ± 83 unit/min/10$^5$ cells, **p<0.01 vs. Ang II, n =23).** **B.** Ang II stimulation increased O$_2^-$ generation in the MMDD1 cells treated with scrambled siRNAs from 853 ± 51 to 2841 ± 83 unit/min/10$^5$ cells (* p<0.01 vs. control). In the MMDD1 cells treated with both Nox2 and Nox4 siRNAs, Ang II-stimulated O$_2^-$ generation was inhibited (# p<0.01 vs. Ang II, n = 15).
Discussion

Ang II, the key effector peptide of renin-angiotensin system, is vital in initiation and progression of hypertension, vascular hypertrophy and atherosclerosis (Kim & Iwao 2000). These actions of Ang II, including vascular smooth muscle constriction, elevated systemic blood pressure, endothelial dysfunction, vascular remodeling, and also retention of sodium, are mediated through the reactive oxygen species (ROS) generated by the Nox (Griendling et al. 1994, Zhang et al. 1999). The ROS are generated following Ang II induced activation of the Ang II type-1 receptors. Indeed, most effects of Ang II in the MD (enhancing TGF, increasing intracellular calcium) are mediated through the AT1 receptors. Ang II influences the $O_2^-$ anion, an important signaling element for hypertension and other deleterious actions of Ang II. We previously used acute doses of Ang II to demonstrate that Ang II increases $O_2^-$ in the MD and the source of this $O_2^-$ is Nox2. These increases in $O_2^-$ enhanced TGF by quenching NO at the MD (Fu et al. 2010, Zhang et al. 2009). It raised the possibility that in situations associated with increased Ang II and oxidative stress like hypertension the increased TGF sensitivity and abnormal sodium handling may be mediated by Rac- enhanced increases in $O_2^-$. However, these studies also used acute Ang II, which does not mimic the pathophysiologic conditions where Ang II is chronically elevated. In this study, we accomplished this by chronically infusing subpressor doses of Ang II. This chronic Ang II induces oxidative stress, salt-sensitive hypertension, increases pro-inflammatory factors and leads to progressive renal injury (Cervenka et al. 2001, Chandrashekar et al. 2012, Chin et al. 1998, Cosentino et al. 1994, Deng et al. 1996, Fitzgerald et al. 1997, Grunfeld et al. 1995, Haas et al. 1999, Laursen et al. 1997, Ortiz et al. 2001a, Ortiz et al. 2001b, Rajagopalan et al. 1996, Reckelhoff et
Indeed, this chronic Ang II model is recognized as a good prototype of human hypertension and found to induce a gradual and sustained elevation in blood pressure over a period of time (Brown et al. 1967, Fu et al. 2010, Romero & Reckelhoff 1999). The dose needed to cause these subpressor Ang II-induced changes vary based on route and species of animals. In mice, it fluctuates anywhere from 400-1000ng/kg/min (Aragon et al. 2008, Brancaccio et al. 2003, Capone et al. 2011, Cassis et al. 2009, Daugherty et al. 2000, Romero & Reckelhoff 1999, Welch et al. 2006). In our study, we used Ang II at a dose of 600 ng/min/kg since we found that this dose consistently elevated MAP by about 20 mmHg. While this dose elevated the blood pressure, it is not as well known whether subpressor Ang II alters oxidative stress at the MD level; and if so, what are the primary sources for Ang II-induced $\text{O}_2^-$ at the MD. Hence, the aim of this study was to determine the changes in the expression as well as function of the two isoforms Nox2 and Nox4 in the MD following chronic Ang II-stimulation. The key findings in our present study are that both Nox2 and Nox4 mRNA levels are increased in the MD following Ang II stimulation and also that the chronic Ang II stimulus enhanced $\text{O}_2^-$ generation from both Nox2 and Nox4. The $\text{O}_2^-$ generated in the MD scavenges NO and plays a vital role in enhancing the TGF response.

Enhanced TGF responses have been found in several models of systemic hypertension. In this study, experiments were performed on isolated perfused juxtaglomerular apparatus and in a MD like cell line, MMDD1 cells to demonstrate that chronic subpressor Ang II elevated $\text{O}_2^-$ in both scenarios. In the isolated perfused JGA form the chronic Ang II treated mice, we noted that chronic low dose Ang II elevated the $\text{O}_2^-$ levels when compared to the controls. This indicates that subpressor Ang II induced
O$_2^-$ in the MD, thereby contributing to the enhanced TGF and Ang II-induced hypertension. In order to determine the possible source of this O$_2^-$, apocynin was used in the JGA preparation. We found that apocynin blunted the O$_2^-$ production in the Ang II animals. We confirmed these observations in in-vitro studies using cultured MMDD1 cells. In these cells, Ang II-induced O$_2^-$ logarithmically increased with increasing Ang II concentrations from 10$^{-10}$ to 10$^{-7}$ log mol/L. The O$_2^-$ production progressively increased from the time of initiating the Ang II stimulation and reached a peak at 12 hrs post Ang II stimulation. This again reaffirms our findings that subpressor Ang II induces O$_2^-$ in the MD of mice. Moreover, the O$_2^-$ from these cells were blunted the most following the addition of apocynin. This suggests that chronic subpressor Ang II increases O$_2^-$ through the Nox system. Also, since apocynin only effectively inhibits Nox2, but not Nox4, it can be hypothesized that in the MD, in a chronic Ang II milieu, the O$_2^-$ may be generated not only from Nox2 but also Nox4. We are aware of the recent publication in which the authors conclude that apocynin may act as a scavenger of reactive oxygen species rather than a specific inhibitor of Nox in some cells (Heumuller et al. 2008). However, this conclusion is primarily based on an experiment in which apocynin at a concentration of 1000 µM reduces the amount of O$_2^-$ in cell-free systems. In addition, 1000 µM apocynin did not have any effect on O$_2^-$ levels in HEK293 cells transfected with Nox2 and p47. These data clearly show that apocynin is not simply a superoxide scavenger. In our preparation we used apocynin at a concentration of 10 µM. Given that this concentration is 100 times less than that needed to scavenge O$_2^-$, it is unlikely that apocynin is acting via this mechanism in our preparation. We also realize that the concentration of oxypurinol we used in this study was pretty high, which could act as an antioxidant (Augustin et al. 1994).
Since xanthine oxidase is not a primary source of $O_2^-$ in the MD, the results will not affect our conclusions for this study.

Our previous studies have demonstrated that the two isoforms of Nox in the MD are Nox2 and Nox4. To confirm this, we used the laser capture microdissection (LCM) technique to isolate the MD as described previously (Zhang et al. 2009). Real Time PCR was utilized to measure the Nox2 and Nox4 mRNA levels. We noted that the Ang II treated mice had about 4 fold increase in Nox2 and 3 fold increase in Nox4 when compared to the control animals. This suggests that both Nox2 and Nox4 isoforms of Nox are increased in chronic Ang II hypertensive mice. Next to isolate which isoform/s of Nox are involved in mediating chronic Ang II induced $O_2^-$ increases, in separate experiments we used silencing RNAs against Nox2 and Nox4 to silence these genes. We found that silencing these genes blunted the effects of chronic Ang II on $O_2^-$ generation; this suggests that unlike acute Ang II stimulation, chronic Ang II stimulates both Nox2 and Nox4 in the MD to generate $O_2^-$. To further test our hypothesis, we used p22phox siRNA to inhibit the function of both Nox2 and Nox4. We found that the subpressor Ang II-induced $O_2^-$ generation in the MMDD1 cells treated with siRNA against p22phox was completely blocked, signifying that both Nox2 and Nox4 are activated by Ang II. Compared to Nox2, there is a paucity of studies defining the significance and mechanism of action of the Nox4 isoform. Some studies on Nox4 suggest that it is the primary source of $O_2^-$ in the thick ascending limbs following acute Ang II stimulation (Massey et al. 2012). Welch et al infused rats with chronic Ang II and measured the SNGFR in rats treated with siRNA against p22phox, and found that the SNGFR in these animals is significantly lowered, implying that Nox plays a very significant role in the regulation of TGF mediated SNGFR.
Moreover, unlike Nox2, Nox4 is also a significant source of hydrogen peroxide, which is essential in regulating vascular activity (Altenhofer et al. 2012, Radermacher et al. 2013, Takac et al. 2012). In our current study we have mainly only focused on the generation of $O_2^-$. Limitations of the present study are that we were unable to perform all the protocols in vivo and in isolated perfused JGAs. We had to use a cell line, namely the MMDD1 cells. We expect that the MMDD1 cells will yield results that are complementary to the results obtained in the perfused JGAs and in vivo. We realize that phenotypically the MMDD1 cell line may not be exactly the same as actual MD cells in vivo (Haas et al. 1999). Any endpoint differences between MMDD1 and actual MD cells should be interpreted with caution.

Although salt sensitivity is considered to be primarily an isolated defect in pressure-natriuresis, along with this deficiency there exists an important physiologic mechanism which plays a part in the renal physiological and structural changes leading to salt sensitivity. This mechanism is known as resetting the set-point of the TGF. In humans resetting the TGF feedback contributes to the development of salt-sensitive hypertension (Aviv et al. 2004, Kotchen et al. 2000, Price et al. 2002). In these hypertensive models, administration of ACE inhibitors or AT1 receptor blockers significantly reduce the sensitivity of the TGF mechanism (Brannstrom et al. 1996, Huang et al. 1988, Mitchell & Mullins 1995, Ploth & Mackenzie 1991), and peritubular infusions of Ang II enhanced the TGF responsiveness (Mitchell & Navar 1988). Thus, Ang II may contribute to the development of hypertension in part through its positive modulating effects on the TGF mechanism.
In summary, we found that a chronic subpressor dose of Ang II enhances $O_2^-$ generation in the MD. The source of this $O_2^-$ is primarily from Nox. Moreover our data also indicate that chronic Ang II stimulates both Nox2 and Nox4.
Chapter Four: Discussion

In the present study, we investigated the pathophysiological mechanisms of the hemodynamic alteration in diabetes. Glomerular hyperfiltration occurs in the early stage of diabetes mellitus and has been recognized to promote the pathogenesis of diabetic nephropathy. We determine the role of TGF response and the vascular tone of Af-Art in diabetic hyperfiltration and we found that 1) tubular high glucose directly activates NOS1 and increases NO generation in the macula densa, which inhibits TGF response and increases GFR; 2) high glucose dilates renal Af-Art through GLUT1 and mediated by NOS3-derived NO generation; 3) in diabetes, blunted TGF mediated by NOS1 in the macula densa promotes glomerular hyperfiltration. The prevalence of hypertension is much higher in diabetic subjects than non-diabetic population. We studied the potential mechanisms of blood pressure regulation in diabetes, including TGF response and renal afferent arteriolar response to vasoconstrictors, and we found that 1) inadequate NOS1 in the macula densa enhances TGF, which restricts glomerular hyperfiltration and induces hypertension in diabetes; 2) hemodynamic responses to ANG II is increased in diabetes, which is associated with increased expression and activity of AT1 receptors in the Af-Art; 3) Ang II upregulates the expression and activity of Nox2 and Nox4 in the macula densa, which enhances TGF response.
References


