Diet-induced prediabetes: Effects on the systemic and renal renin-angiotensin-aldosterone system

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Research Article

Keywords: Renin-angiotensin-aldosterone system, diet-induced prediabetes, high-fat high carbohydrate diet, renal failure

DOI: https://doi.org/10.21203/rs.3.rs-120506/v1

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Abstract

The activity of the renin-angiotensin-aldosterone system (RAAS) in type 2 diabetes (T2D) has been characterized. However, the effects of high-fat high carbohydrate diet-induced prediabetes on the RAAS has not been elucidated. Hence, male Sprague Dawley rats were randomly assigned to a normal diet (NPD) group and (HFHC) group (n=6) for 20 weeks to allow for the induction of prediabetes. Blood glucose concentration, mean arterial pressure (MAP), kidney renin, angiotensinogen, angiotensin-converting enzyme (ACE), angiotensin II type 1a receptor (Agtr1a) in addition to kidney and plasma angiotensin II (Ang II), aldosterone were analyzed at week 20 to investigate the RAAS activity. In addition to kidney injury marker (Kim1) and urinary protein, concentrations were analyzed at week 20. The results demonstrated an increase in blood glucose, MAP, relative expression of kidney RAAS components in the HFHC group by comparison to the NPD. Furthermore, an increase in plasma Ang II and aldosterone was accompanied by elevated Kim-1 and albumin excretion in the HFHC diet group by comparison to the NPD group. Thus, we suggest that the RAAS is activated in diet-induced prediabetes and may induce early kidney damage.

Introduction

The increased consumption of high caloric diets has been associated with an increased prevalence of type 2 diabetes and prediabetes [1,2]. Prediabetes is a state in which the blood glucose concentration is above the homeostatic range yet below the threshold for diagnosis of clinical diabetes, indicative of a high risk of progression to type 2 diabetes mellitus [3,4]. The International Diabetes Federation (IDF) estimates that 3.85 million South Africans between the ages of 21 and 79 years may have diabetes currently [5]. While it estimates that a further 5 million are prediabetic. A positive correlation has been noted between T2D, dyslipidemia, altered energy metabolism, dysregulated insulin signaling, inflammation, endoplasmic reticular stress, oxidative stress, and the upregulation of the renin-angiotensin-aldosterone system (RAAS) [6].

The renin-angiotensin-aldosterone system (RAAS) is a regulatory signaling pathway that maintains blood pressure through the regulation of fluid and electrolyte homeostasis [7]. Researchers have demonstrated that in T2D, RAAS is upregulated, resulting in oxidative stress, renal failure, hypertension, and cardiac dysfunction. In our laboratory, we created a diet-induced prediabetes animal model that mimics metabolic processes that occur in humans during the prediabetes stage [8,9]. Several studies conducted on this model have illustrated that changes observed in T2D often begin during the prediabetic state [10]. While the alterations that occur to the renin-angiotensin-aldosterone system in the diabetic state have been well documented, the changes that occur in RAAS in prediabetes are unknown [11]. Hence, in this study, we used our diet-induced prediabetic rat model to investigate the alterations that occur in the renin-angiotensin-aldosterone system during the prediabetic state.

Results
OGTT response and plasma insulin

An OGTT was conducted on the non-pre-diabetic (NPD) and the prediabetic (PD) at week 20 whereby, the NPD and PD were exposed to 12 hours fasting, and glucose was measured followed by the oral administration of monosaccharide syrup (glucose;0.86 g/kg, p. o) at different time intervals (15, 30, 60 and 120 minutes) to assess glucose tolerance. The results (Fig 1a) revealed that the blood glucose concentration at time 0 the PD had a significantly (p<0.05) higher blood glucose concentration when compared to NPD, and the same trend was observed at time 15, 30, 60 and 120. Furthermore, the (Fig 1b) plasma insulin concentrations were significantly (p<0.0001) higher in the PD group by comparison to the non-diabetic group.

Mean arterial pressure

The mean arterial pressure (MAP) of the NPD and PD was measured at week 20. The results (Fig 2) revealed that the mean arterial pressure levels were significantly (p<0.0001) higher in the PD group in comparison to the non-pre-diabetic group.

Plasma angiotensin 2

The plasma angiotensin 2 (Ang II) of the NPD and PD was measured at week 20. The results (Fig 3) revealed that the plasma angiotensin 2 was significantly (p=0.02) higher in the PD group in comparison to the non-pre-diabetic group.

Plasma aldosterone

Plasma aldosterone was measured for both the PD and NPD group at week 20. The results (Fig 4) showed that the plasma aldosterone concentration was significantly (p=0.0001) higher in the PD group in comparison to the NPD group.

Oxidative stress markers

The kidney NADPH oxidase, MDA, SOD, and GPx1 of the NPD and PD was monitored at week 20. The results (Fig 5) revealed that the NADPH oxidase and MDA concentration was significantly (p≤0.0001), (p=0.0072), (p=0.0015), and (p=0.0072) higher in the PD group in comparison to the NPD whilst the SOD and GPX1 were significantly lower in the PD by comparison to the NPD group.

Kidney RAAS components

The kidney renin, angiotensinogen (Agt), ACE, and Agtr1a of the NPD and PD were monitored at week 20. The results (Fig 6) revealed that the relative expression of these RAAS components was significantly (p=0.04) and (p<0.001) higher in the PD group relative to the non-prediabetic group.

Kidney angiotensin II and aldosterone
The kidney Ang II and aldosterone of the NPD and PD groups were monitored at week 20. The results (Fig 7) revealed that the kidney Ang II and aldosterone were significantly (p=0.01) higher in the PD group by comparison to the non-prediabetic group.

**Plasma Kidney injury marker**

Kidney injury marker (Kim1) concentrations were measured in the plasma of non-pre-diabetic (NPD) and prediabetic (PD) rats at week 20. The results (Fig 8) revealed that the concentration of Kim1 was significantly (p=0.04) higher in the PD group in comparison to the non-diabetic group.

**Urinary protein ratio (UPC)**

The urinary protein creatinine ratio of the non-pre-diabetic (NPD) and prediabetic (PD) was measured at week 20. The results (Fig 9) revealed that the urine protein ratio concentration was significantly (p=0.0013) higher in the PD group by comparison to the non-diabetic group.

**Discussion**

In the present study, there was an upregulation of the renin-angiotensin-aldosterone system's systemic and local components, namely; renin, Ang II, aldosterone, and the expression of the angiotensin II type 1a receptor (Agtr1a). The upregulation of RAAS contributed to renal system impairment, which was evidenced by proteinuria and the significantly increased concentration of the kidney injury marker (Kim 1). Oxidative stress was observed in the kidney, which was evidenced by the increased MDA and NADPH oxidase and decreased antioxidants (SOD1 and GPx1) in the prediabetic group by comparison to the non-pre-diabetic group.

Researchers have demonstrated a positive correlation between insulin resistance and hypertension [12]. In the current study, the mean arterial pressure was elevated during the prediabetic state [13]. This elevation may be a consequence of the hyperinsulinemia and insulin resistance noted in prediabetes [13]. Insulin is responsible for the vasodilation of coronary resistant vessels, in turn regulating sympathetic nerve activity [14]. The hyperinsulinemia and insulin resistance in prediabetes intensifies the sympathetic activity, thus contributing to the elevation of the mean arterial pressure (MAP) [14]. Additionally, in vascular endothelial cells, insulin prompts the synthesis of nitric oxide via the protein kinase B pathway [15]. In the insulin-resistant state, the stimulation of the insulin receptor substrate (IRS)-1/PI-3 kinase pathway by insulin is severely impaired, causing a decrease in NO, which is a potent vasodilator; consequently, the mean arterial pressure is increased, which if untreated, could result in the development of hypertension [16,17]. In this study, a notable increase in plasma angiotensin 2 and aldosterone concentration was observed. We speculate that this rise could be a result of the upregulation of RAAS as these hormones are products of the activity of this system and are pivotal in electrolyte homeostasis, thus affecting the mean arterial pressure [18]. Aldosterone acts on the cells of the collecting ducts and induces expression of Na⁺/K⁺ exchangers, which promote sodium retention and potassium excretion,
consequently increasing blood volume and pressure\textsuperscript{[19]}. An increase in angiotensin 2 and aldosterone concentration observed in this study, suggesting an activation of RAAS in a pre-diabetic state which could in part explain the elevation of MAP observed.

The renin-angiotensin-aldosterone system (RAAS) is an important pathway implicated in the pathogenesis and progression of diabetic nephropathy\textsuperscript{[20,21]}. Ang II is a primary hormone of the RAAS and contributes to a variety of renal and cardiovascular physiologic and pathological mechanisms\textsuperscript{[20,22,23]}. Some authors have found that Ang II in the kidney generates reactive oxygen species (ROS) and promotes podocyte autophagy by enhancing the podocyte expression of autophagic genes, LC3-2, and beclin-1\textsuperscript{[24-27]}. Angiotensin 2 impairs the insulin signaling pathway by hindering the phosphorylation of insulin receptor substrate (IRS)-1 in the muscle\textsuperscript{[28,29]}. Consequently, phospho-inositol 3 kinase remains inactive; therefore, Akt, which is responsible for the translocation of GLUT-4 vesicles, is also inactive; hence plasma glucose is not transported into the cell to be converted by glycogen synthase to glycogen\textsuperscript{[18,30]}. Through this action, we speculate that the activated renin-angiotensin-aldosterone system contributes to insulin insensitivity and hyperglycemia, which was observed in the present study\textsuperscript{[18,31]}. Angiotensin 2 activates nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, which transfers electrons from nicotinamide adenine dinucleotide (NADPH) inside the cell, generating superoxide which reacts with coupled molecular oxygen generating reactive superoxide anion and hydrogen peroxide resulting in the generation of reactive oxygen species which accumulate and cause oxidative stress which decreases insulin-dependent GLUT-4 translocation\textsuperscript{[31-33]}. In the present study, moderate hyperglycemia and an imbalance in the reactive oxygen species, which was measured by the MDA concentration and antioxidants, SOD1 and GPX1, were observed. We can then speculate that the activation of the renin-angiotensin-aldosterone system (RAAS) in the prediabetic state does not only affect the insulin signaling pathway but also affects various organ systems such as the renal and circulatory system through instigation of oxidative stress\textsuperscript{[34,35]}. The increase in glucose load, which is partly attributable to the impaired insulin signaling pathway, has been proven to have proapoptotic effects on podocytes in a hyperglycaemic state\textsuperscript{[36]}. The proapoptotic effect of high glucose in podocytes is mediated by activation of NADPH oxidase, which is followed by the generation of ROS, promoting lipid peroxidation and a decrease in the antioxidant's levels, namely; GPX1 and SOD, which is consistent with the increased kidney NADPH oxidase, MDA and decreased GPX1 and SOD1 concentration in this study which we postulate results in apoptosis of the podocyte, therefore, causing structural changes in the kidney which might contribute to renal failure if untreated. Furthermore, NADPH oxidase activates the poly (ADP-ribose) polymerase, NFkB, and p38 MAP kinase (Susztak et al., 2006; Szabo et al., 2006;\textsuperscript{[36]}). In addition to its direct effects, elevated glucose may act indirectly, via the Ang II-dependent pathways\textsuperscript{[37]}. The hyperglycemia noted in T2D has been proven to result in an increase in the citric acid cycle intermediate, succinate\textsuperscript{[38]}. The Succinate-GPR91 receptor activation precipitates elevation in cytosolic Ca\textsuperscript{2+}, nitric oxide, and prostaglandin bioavailability\textsuperscript{[39,40]}. Prostaglandin acts on the juxtaglomerular renin producing cells and triggers a cascade that results in renin release,
angiotensinogen, angiotensin 1 and 2 syntheses. Furthermore, nitric oxide and prostaglandin stimulate vasodilation of the afferent arteriole, thus enabling increased blood flow in the glomerulus. Interestingly the produced Ang II triggers vasoconstriction of the efferent artery, thus increasing the glomerular filtration rate and inherently contributing to the glomerular filtration barrier injury, which may result in the passage of solutes such as small proteins resulting in microalbuminuria. In in vivo, as well as in vitro experiments, Yoo et al. (2007) demonstrated that in podocytes, hyperglycemia, similarly to mechanical stress, stimulates activity and expression of the local RAS components, including Ang II and its AT1 receptors. Since Ang II, via induction of NADPH oxidase, promotes the production of ROS, this may be the mechanism contributing to the proapoptotic effects exerted by Ang II on podocytes.

Moreover, TGFB, a proapoptotic cytokine whose activity is induced by hyperglycemia and, most likely, by mechanical stress, is also activated by Ang II. In this regard, Ang II may be considered as an additional factor that potentiates the effects of high glucose and mechanical stress in podocytes. The upregulated renin-angiotensin-aldosterone system in diabetes has been proven to result in renal failure, possibly by increasing aldosterone concentration, which promotes sodium retention and potassium excretion, consequently resulting in increased systemic and glomerular pressure. These findings are consistent with our observed changes in RAAS components in the kidney and the elevated mean arterial pressure. This is in addition to the increased oxidative stress, which has been reported to cause kidney failure in T2D, resulting in proteinuria, which was also supported by increased levels of Kim1 and the reduced urinary creatinine concentration demonstrated by the decreased urinary protein ratio.

Conclusion

Taken together, the results described in the present study suggest that the moderate hyperglycemia noted in diet-induced prediabetes activates the renin-angiotensin-aldosterone system, which might contribute to the complications seen in type 2 diabetes such as insulin resistance, hypertension, and renal system impairment. Future studies are, however, required to investigate the exact mechanisms that contribute to these changes.

Methods And Materials

Animals and housing

Male Sprague-Dawley rats (150-180g) bred and housed in the Biomedical Research Unit (BRU) of the University of KwaZulu-Natal were used in the study. The animals were maintained under standard laboratory conditions of temperature in the range of (22±2 °C), CO₂ content (<5000 p.m.), relative humidity (55±5%), and illumination (12 h light/dark cycle, lights on at 07h00). The noise level was maintained at less than 65 decibels as described by Luvuno et al., 2020. The animals were allowed access to food and fluids ad libitum. All animal experimentation was approved by the Animal Research Ethics Committee of the University of KwaZulu-Natal (ETHICS #: AREC/024/0180). The animals were allowed to acclimatize to their new environment for 1 week while consuming standard rat chow and tap
water before exposure to the experimental diets [50]. Procedures involving animal care were conducted in conformity with the institutional guidelines for animal care of the University of KwaZulu-Natal in conjunction with the ARRIVE guidelines.

**Induction of prediabetes**

The animals were randomly assigned to the following diet groups: standard rat chow with normal drinking water (NPD, n=6) and high-fat high-carbohydrate (HFHC) diet with drinking water supplemented with 15% fructose (HFHC+Fructose, n=6) (AVI Products (Pty) Ltd, Waterfall, South Africa). Prediabetes was induced by allowing the animals to feed on the HFHC and fructose diet for 20 weeks, as previously described by Luvuno et al., 2018 [8]. After 20 weeks, the American Diabetes Association criteria were used to diagnose prediabetes whereby the criteria to define prediabetes include impaired fasting glucose (IFG) with fasting plasma glucose levels of 5.6 to 6.9 mmol/L, impaired glucose tolerance (IGT) with plasma glucose levels of 7.8 to 11.0 mmol/L 2-hour postprandial, or glycated hemoglobin (HbA1c) of 5.7 to 6.4%. The animals that were fed the standard diet were also tested at week 20 and were found to be normoglycemic and without prediabetes.

**OGTT response**

The NPD and the PD groups were exposed to an 18-hour fasting period. At the end of the fasting period, their blood glucose was measured (time 0) immediately followed by loading with a monosaccharide syrup (glucose; 0.86 g/kg, p.o.) by oral gavage using an 18-gauge gavage needle that is 38 mm long curved, with a 21/4 mm ball end (Able Scientifc, Canning Vale, Australia) [51]. Blood was collected via the tail pick method using a OneTouch select glucometer (Lifescan, Mosta, Malta, United Kingdom) to determine the blood glucose concentration at times 0, 15, 30, 60, 90, and 120 minutes.

**Blood pressure measurements**

The systolic, diastolic, and mean arterial blood pressure (MAP) was measured at week 20 using the non-invasive tail-cuff method with photoelectric sensors (IITC Model 31 Computerised Blood Pressure Monitor, Life Sciences, Woodland Hills, California, USA) as previously described by Gamede et al., 2019 [52]. The equipment was calibrated each day prior to measurements. The animals were kept warm at ± 30°C in an enclosed chamber (IITC Model 303sc Animal Test Chamber, IITC Life Sciences, Woodland Hills, California, USA) for 30 minutes before blood pressure recording. All measurements were conducted at 09h00 as previously described [52].

**Blood collection and tissue harvesting**

At the end of the experimental period, all animals were anesthetized with isoflurane (100 mg/kg) (Isofur, Safeline PharmAceuticals (Pty) Ltd, Roodeport, South Africa) via a gas anesthetic chamber (Biomedical Resource Unit, UKZN, Durban, South Africa) for 3 minutes. While rats were unconscious, blood was collected by cardiac puncture and then injected into individual pre-cooled heparinized containers. The
blood was then centrifuged (Eppendorf centrifuge 5403, Germany) at 4°C, 503 g for 15 minutes. Plasma was collected and stored at -70 °C in a Bio Ultra freezer (Snijers Scientific, Holland) until biochemical analysis as previously described by Luvuno et al., 2020\textsuperscript{50}.

**Biochemical analysis**

The kidney NADPH oxidase, SOD, GPX1, and Kim1 as well as plasma insulin, Ang II, and aldosterone concentration were measured using separate, specific ELISA kits according to the manufacturer's instructions (Elabscience and Biotechnology, Wuhan, China). MDA concentration was determined using a previously described protocol by Mkhwanazi et al., 2014\textsuperscript{53}.

**Quantitative real-time PCR**

RNA extraction in the kidney was performed as per the ReliaPrep miRNA Cell and Tissue Miniprep System (Promega, USA). Synthesis of cDNAs was performed by reverse transcription reactions with 2 μg of total RNA using GoTaq® 2-Step RT-qPCR System as a cDNA synthesis kit (Promega, USA) as described by the manufacturer.

The ROCHE light cycler SYBR Green I master mix was used for amplification according to the manufacturer's instructions on the ROCHE light cycler system. The primer sequences (Metabion, Germany) used in this study can be found in Table 1 below. The cycling conditions were: Pre-incubation was carried out at 95°C for 60s, followed by a 3-step amplification of 45 cycles at 95°C for 15s, 60°C for 30s, and 72°C for 30s. Melting was effectuated at 95°C for 10s, 65°C for 60s and 97°C for 1s. Furthermore, cooling was achieved at 37°C for 30s. Glyceraldehyde-phosphate dehydrogenase (GAPDH) as an internal control was used as the housekeeping gene. Gene expression values are represented using the $2^{-\Delta\Delta Ct}$ relative quantification method.

**Table 1**: List of primers used in this study

Table 1: List of primers used
| Gene of interest | Sequence |
|------------------|----------|
| renin            | Forward: 5′-GAGG-CCTCCTTGACCAATC- 3′  
Reverse: 5′- TGT-GAATCCACAAGCAAG-3′ |
| angiotensinogen  | Forward: 5′- GAGTGGGAGAGGTTCTC-AA-3′  
Reverse: 5′- TCGTAGATGCGAAGCAG-GA-3′ |
| ACE              | Forward: 5′- CCATCTGCTAGGGAA-CATGT-3′  
Reverse: 5′- GTGTCCATCCCTG-CTTTATCA-3′ |
| AT1R             | Forward: 5′- GCTCACGTG-TCTCAGCAT-3′  
Reserve: 5′- TTGGCCAC-CAGCATCGT-3′ |
| GAPDH            | Forward: 5′-AGTGCCAGCCTCGTCTCATA-3′  
Reserve: 5′-GATGGTGATGGGTTTCCCGT-3′ |

The urine creatinine ratio was determined using IDEXX vetlab station (USA IDEXX Laboratories, Inc). One IDEXX Drive Westbrook, ME 04092 USA 1-800-548-6733.

**Analysis of data**

All data were expressed as means ± S.E.M. Statistical comparisons were performed with Graph Pad instat Software (version 5.00, GraphPad Software, Inc., San Diego, California, USA) using Student's unpaired two-sided t-test. A value of p< 0.05 was considered statistically significant.

**Declarations**

**Acknowledgments**

The authors are grateful to Mr. Makhubela for his technical expertise, the staff of the Biomedical Resource Unit, the University of KwaZulu-Natal for the supply of animals, and the College of Health Sciences (CHS).

**Ethics approval**

All animal experimentation was approved by the Animal Research Ethics Committee of the University of KwaZulu-Natal (Ethical clearance number: AREC/024/0180).

**Consent for publication**

Not applicable
Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Disclosure of interest

The authors report no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by the college of health science (CHS) of the University of KwaZulu-Natal (UKZN).

Author contributions

BCM and PM were involved in study design, conducted the experiments, analyzed and interpreted data, and was involved in writing the manuscript. PSN and NHS were involved in the study design, analyzed and interpreted data, and was involved in writing the manuscript. AK was involved in the conceptualization of the study; study design provided funding, analyzed and interpreted data, and was involved in the writing of the manuscript. All authors have read and approved the final manuscript.

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Figures
Figure 1

An OGTT at week 20 was conducted in addition to the analysis of the plasma insulin concentration on the non-prediabetic (NPD) n=6 and pre-diabetic (PD) n=6. The glucose and plasma insulin concentration was significantly higher in the PD by comparison to the NPD. a) blood glucose and b) plasma insulin concentrations of non-pre-diabetic and pre-diabetic rats. Values are presented as means ± SEM. a) *p<0.05 and b) ***p<0.0001 denotes comparison with NPD male Sprague Dawley rats.

Figure 2
The mean arterial pressure of non-prediabetic (NPD) n=6 and pre-diabetic (PD) n=6 male Sprague Dawley rats was measured at week 20 and was found to be significantly higher in the PD by comparison to the NPD. Values are presented as means ± SEM. *p=0.0001 denotes comparison with NPD male Sprague Dawley rats.

**Figure 3**

The plasma angiotensin II (Ang II) concentration of the non-prediabetic (NPD) n=6 and pre-diabetic (PD) n=6 was measured at week 20 where the PD had a significantly higher concentration by comparison to the NPD. Values are presented as means ± SEM. *p=0.02 denotes comparison with NPD male Sprague Dawley rats.
The plasma aldosterone concentration of the non-prediabetic (NPD) n=6 and pre-diabetic (PD) n=6 was measured at week 20 where the PD had a significantly higher concentration by comparison to the NPD. Values are presented as means ± SEM. ★★★p=0.0001 denotes comparison with NPD male Sprague Dawley rats.
Figure 5

The oxidative stress of the non-prediabetic (NPD) n=6 and pre-diabetic (PD) n=6 was determined at week 20 through the analysis of NADPH Oxidase, Malondialdehyde (MDA), Superoxide dismutase (SOD) and Glutathione (GPx1). There was a notable increase in the NADPH Oxidase and lipid peroxidation determinant (MDA) and decrease in the concentration of antioxidant enzymes (SOD, GPx1) in the prediabetic control rats by comparison to the non-prediabetic control rats. a) NADPH oxidase, b) MDA and c) SOD and d) GPx1 concentration of rats. Values are presented as means ± SEM. a) \( p \leq 0.0001 \), b) \( p=0.0072 \), c) \( p=0.0015 \) and d) \( p=0.0072 \) denotes comparison with NPD male Sprague Dawley rats.
The relative expression of the kidney RAAS components viz; a) Renin. b) Angiotensinogen, c) Angiotensin-converting enzyme (ACE) and d) Angiotensin II type 1 receptor (Agtr1a) of the non-prediabetic (NPD) n=6 and pre-diabetic (PD) n=6 was measured at week 20. The relative expression of renin, angiotensinogen, Ace and Agtr1a were significantly higher in the PD by comparison to the NPD. Values are presented as means ± SEM. a) $p=0.0001$, b) $p=0.0124$ c) $p=0.0004$ and d) $p=0.0006$ denotes comparison with NPD male Sprague Dawley rats
Figure 7

The plasma concentration of the kidney a) angiotensin II (Ang II) and b) aldosterone of the non-prediabetic (NPD) n=6 and pre-diabetic (PD) n=6 was measured at week 20. The concentrations of the kidney Ang II and aldosterone were significantly in the PD by comparison to the NPD. Values are presented as means ± SEM. a) $p=0.0032$ and b) $p=0.0019$ denotes comparison with NPD male Sprague Dawley rats.

Figure 8

The kidney injury marker 1 (Kim-1) concentration of the non-prediabetic (NPD) n=6 and pre-diabetic (PD) n=6 was measured at week 20 where the PD had a significantly higher concentration by comparison to
the NPD. Values are presented as means ± SEM. *p=0.0001 denotes comparison with NPD male Sprague Dawley rats

Figure 9

The urine protein creatinine ratio (UPC) of the non-prediabetic (NPD) n=6 and pre-diabetic (PD) n=6 was measured at week 20 where the PD had a significantly higher UPC ratio by comparison to the NPD. Values are presented as means ± SEM. *p=0.0013 denotes comparison with NPD male Sprague Dawley rats.