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## Urinary angiotensinogen increases in the absence of overt renal injury in high fat diet-induced type 2 diabetic mice

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### Abstract

**Aim of the Study:** During type 2 diabetes (T2D) and hypertension there is stimulation of renal proximal tubule angiotensinogen (AGT), but whether urinary excretion of AGT (uAGT) is an indicator of glomerular damage or intrarenal RAS activation is unclear. We tested the hypothesis that elevations in uAGT can be detected in the absence of albuminuria in a mouse model of T2D.

**Methods:** Male C57BL/6 mice (N=10) were fed a high fat (HFD; 45% Kcal from fat) for 28 weeks, and the metabolic phenotype including body weight, blood pressures, glucose, insulin, ippgTT, HOMA-IR, and cholesterol was examined. In addition, kidney Ang II content and reactive oxygen species (ROS) was measured along with urinary albumin, creatinine, Ang II, and AGT.

**Results:** All parameters consistent with T2D were present in mice after 12–14 weeks on the HFD. Systolic BP increased after 18 weeks in HFD but not NFD mice. Intrarenal ROS and Ang II concentrations were also increased in HFD mice. Remarkably, these changes paralleled the augmentation uAGT excretion ( $3.66 \pm 0.50$  vs.  $0.92 \pm 0.13$  ng/mg by week 29;  $P < 0.01$ ), which occurred in the absence of overt albuminuria.

**Conclusions:** In HFD-induced T2D mice, increases in uAGT occur in the absence of overt renal injury, indicating that this biomarker accurately detects early intrarenal RAS activation.

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Disclosures  
None

## Keywords

Intrarenal Renin-Angiotensin System; Albuminuria; Kidney; Hyperglycemia; Diabetes Mellitus; Blood pressure

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## 1. INTRODUCTION

The systemic renin-angiotensin system (RAS) is a key regulator of blood pressure (BP) and fluid and electrolyte homeostasis. The intrarenal RAS also plays a critical role in the regulation of kidney function, but when stimulated inappropriately promotes hypertension and renal disease. During angiotensin (Ang) II-dependent hypertension, higher levels of renal versus circulating Ang II indicate activation of the intrarenal RAS<sup>1</sup>. In contrast, RAS blockade reduces intrarenal RAS activation<sup>2,3</sup> in hypertensive as well as diabetic humans and rodents<sup>4,5</sup>. Angiotensinogen (AGT), the key renin substrate in the formation of Ang II, is primarily formed and secreted by the liver but is also synthesized and secreted by the proximal tubule<sup>6,7</sup>. Hypertension<sup>8,9</sup> and type 2 diabetes<sup>10,11</sup> increase intrarenal AGT transcription and protein in both humans and rodents. Indeed, urinary AGT levels (uAGT) are used as biomarker of intrarenal RAS activation and positively correlates with renal Ang II in both, humans<sup>8,12</sup> and experimental animal models<sup>13</sup>. However, in subjects with renal damage<sup>14</sup>, uAGT highly correlates with albuminuria<sup>15</sup>, suggesting that it may be an indicator of glomerular damage rather than intrarenal RAS activation<sup>16</sup>. Nevertheless, the lack of evidence of temporal changes of uAGT during the development of type-2 diabetes (T2D) has contributed to the uncertainty about the relationship between intrarenal RAS activation and the development of renal disease during T2D.

This study aimed to discriminate whether increases in uAGT could be dissociated from albuminuria and to understand the ability of uAGT to predict early intrarenal RAS activation in a mouse model of high fat diet (HFD)-induced T2D with slow development of renal disease. We tested the hypothesis that intrarenal RAS activation as reflected by increased uAGT excretion could be detected in the absence of the development of albuminuria in mice with HFD-induced T2D.

## 2. MATERIALS AND METHODS

### 2.1 Development of a high fat diet induced-type 2 diabetes (HFD-T2D) mouse model

All animal procedures were approved by the Tulane University Animal Care and Use Committee. Male C57BL/6 mice 3 weeks-old, were weaned and kept under standard conditions (12-h light/dark cycles and  $21 \pm 2^\circ\text{C}$ ). All the mice were  $4 \pm 1-2$  weeks of age (N=20) where the study started. Samples of blood and urine were collected to measure baseline parameters (week 0). Due to the young age of mice to withstand the stress while in metabolic caging, we placed each mouse on hydrophobic sand (LabSand, Coastline Global Inc, Palo Alto, CA) using a single cage to collect urine samples according to the manufacturer's instructions. At 5 weeks of age, mice (N=20) were randomly divided into 2 groups, based on dietary regimen: 1) Normal fat diet (NFD; N=10) and 2) High fat diet (HFD; N=10). The NFD (PicoLab® Rodent Diet 20 EXT IRR 5R53, St. Louis, MO)

consisted of 25% Kcal from protein, 13% from fat, and 62% from carbohydrate. The HFD (TestDiet, DIO 58V8–45, St. Louis, MO) contained 18% Kcal from protein, 45% from fat, and 36% from carbohydrate. Two separate sets of mice were used: one for metabolic purposes (N=6 mice per group) and other for blood pressure recordings using radiotelemetry (N=4 mice per group). Food and water were *ad libitum* during the following 28 weeks, until the study was completed.

## 2.2 General Experimental Protocols

After 12 hours of fasting, blood samples were collected by submandibular plexus bleeding in EDTA (5mmol/L) chilled tubes. Plasma was obtained by centrifugation at  $3500 \times g$  at  $4^{\circ}\text{C}$  for 15 min. Urine samples were collected from mice individually housed in metabolic cages and centrifuged at  $3500 \times g$ ,  $4^{\circ}\text{C}$  for 30 min to remove particulate. Urine and blood samples were collected every four weeks. At the study endpoint, mice were euthanized under deep isoflurane inhalation anesthesia and tissues were harvested, weighed, snap frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ .

## 2.3 Assessment of the HFD-induced type 2 diabetes mouse model:

**2.3.1 Body weight and metabolic measurements:** Body weights (BW) from mice fed either a NFD or HFD were measured every 4 weeks with accuracy within 0.1 g. To obtain the daily energy intake per mouse, food intake was multiplied by the number of Kcal provided per gram of food (NFD: 4.07 Kcal/g of food and HFD 4.60 Kcal/g of food)<sup>17</sup>. Blood glucose levels were monitored monthly, from a drop of blood obtained by milking tail and measured with a hand-held glucometer (ONETOUCH Ultra glucometer, LifeScan, catalog #ZJZ8158JT, Milpitas, CA). Intraperitoneal glucose tolerance tests (ipGTT) were performed monthly. Blood glucose concentrations were measured before (0 min) and after (15, 30, 60 and 120 min) a glucose overload (2 g/kg BW, i.p.)<sup>18</sup>. Plasma cholesterol and triglyceride concentrations were measured using a Wako Kit (Wako Diagnostics, catalog #439–17501 and 464–016001, respectively, Richmond, VA). Plasma insulin concentrations were quantified by ELISA (Rat/Mouse Insulin assay Kit, EMD Millipore, catalog #EZRMI-13K, Billerica, MA). Insulin resistance (IR) was calculated by the homeostasis model assessment index as  $\text{HOMA-IR} = (\text{Insulin} \times \text{Glucose}) / 22.5$ .

**2.3.2 Diastolic, Systolic and Mean Arterial Pressures:** Mice were anesthetized by isoflurane inhalation and a left carotid catheter connected to a radiotelemetry transmitter was implanted (TA11-PAC10, Data Sciences Inc., Palo Alto, CA). Pressures were recorded in conscious mice after 2 weeks of recovery for one 24 h period each week for four weeks using Ponemah 6.0 software.

## 2.4 Angiotensinogen excretion in urine

Angiotensinogen concentration in urine (uAGT) was measured by ELISA (Mouse Total Angiotensinogen Assay Kit, IBL, catalog #27413, Japan). This assay was developed and optimized previously in our department<sup>19</sup>. Urinary excretion rates of uAGT were calculated from the 12 h volumes collected.

## 2.5 Albumin and creatinine in urine

Mouse albumin in urine was measured by ELISA (Abcam, catalog #108792, Billerica, MA)<sup>19</sup>. Creatinine concentrations, measured by colorimetric assay (QuantiChrom™ Creatinine Assay Kit, BioAssay Systems, Hayward, CA), were used to calculate the urinary albumin/creatinine ratio (UACR) in order to correct for variations in urine concentration due to the degree of hydration.

## 2.6 Angiotensin II in kidney and urine

AT THE END OF THE STUDY, URINE SAMPLES WERE COLLECTED INTO TUBES CONTAINING AN INHIBITOR COCKTAIL (5 MMOL/L OF EDTA, 20 μMOL/L OF PEPSTATIN A, 10 μMOL/L OF PMSF, 20 μMOL/L OF ENALAPRILAT, AND 1.25 MMOL/L OF 1,10-PHENANTHROLINE). LEFT KIDNEYS WERE DECAPSULATED AND IMMERSSED IN COLD 100% METHANOL. AFTER HOMOGENIZATION, CENTRIFUGATION AND EVAPORATION THE SAMPLES WERE EXTRACTED AND EVAPORATED AS DESCRIBED PREVIOUSLY<sup>20</sup>. ANGIOTENSIN II CONCENTRATIONS WERE MEASURED BY EIA (ANGIOTENSIN II EIA KIT, CAYMAN, CATALOG #A05880, MONTIGNY-LE-BRETONNEUX, FRANCE) AND EXPRESSED PER GRAM OF WET WEIGHT. URINARY EXCRETION RATES WERE CALCULATED FROM THE 12 H VOLUMES COLLECTED.

## 2.7 Fibrotic factors protein levels in the kidney

Protein expression of several fibrotic factors including ALPHA-smooth muscle actin (α-SMA), fibronectin, collagen I, and transforming growth factor-β (TGF-β) were measured by Western blot of whole kidney lysates and normalized to β-actin, as previously reported<sup>21</sup>.

## 2.8 Reactive oxygen species (ROS) in the kidney

SUPEROXIDE PRODUCTION WAS QUANTITATIVELY MEASURED BY ELECTRON SPIN RESONANCE (ESR) SPECTROSCOPY USING THEE MODIFIED METHOD PREVIOUSLY REPORTED<sup>22</sup>. THE SUPEROXIDE-SPECIFIC SPIN PROBE 1-HYDROXY-3-METHOXYCARBONYL-2,2,5,5-TETRAMETHYL-PYRROLIDINE (CMH) WAS USED TO IDENTIFY AND QUANTITATE SUPEROXIDE LEVELS (NOXYGEN GmbH, ELZACH, GERMANY). DIETHYLDITHIOCARBAMATE (DETC; 2.5 μMOL/L) AND DESFEROXAMINE (DF; 25 μMOL/L) WERE DISSOLVED UNDER NITROGEN GAS BUBBLING IN ICE-COLD MODIFIED KREBS-HEPES (KH) BUFFER CONTAINING (IN MMOL/L) 99.01 NaCl, 4.69 KCl, 1.87 CaCl<sub>2</sub>, 1.20 MgSO<sub>4</sub>, 25 NaHCO<sub>3</sub>, 1.03 K<sub>2</sub>HPO<sub>4</sub>, 20 Na-HEPES, AND 11.1 D-GLUCOSE, AT pH 7.35. KIDNEY TISSUE WAS CUT INTO 3–5 MM PIECES AND PLACED INTO WELLS OF A 96-WELL PLATE CONTAINING KH BUFFER (200 ML/WELL) WITH FRESHLY MADE CMH (200 μMOL/L), DETC, AND DF FOR 60 MIN AT 37°C. FIRST, 300 μL KH BUFFER WAS TRANSFERRED TO 1 ML SYRINGE, SNAP FROZEN IN LIQUID NITROGEN. SECOND, 200 ML OF KH BUFFER CONTAINING THE TISSUE SAMPLE WAS TRANSFERRED TO THE SAME SYRINGE BEFORE SNAP FREEZING AGAIN. THE TWO-LAYERED SAMPLES WERE TRANSFERRED TO A FINGER DEWAR FILLED WITH LIQUID NITROGEN AND INSERTED INTO THE EMX ESR eSCAN BENCHTOP SPECTROMETER (BRUKER, GERMANY). ESR SPECTRA OF SAMPLES WERE ACQUIRED USING THE FOLLOWING ESR SETTINGS: FIELD SWEEP, 80 G; MICROWAVE FREQUENCY, 9.39 GHz; MICROWAVE POWER, 2 mW; MODULATION AMPLITUDE, 5 G; CONVERSION TIME, 327.68 MS; TIME CONSTANT, 5242.88 MS; 512 POINTS RESOLUTION AND RECEIVER GAIN, 1×10<sup>3</sup>. THE AMPLITUDE MEASUREMENTS OF THE ESR SPECTRA IN ARBITRARY UNITS WERE NORMALIZED TO THE DRY WEIGHT OF THE CORRESPONDING TISSUE SAMPLES.

## 2.9 Statistical analyses

Results are expressed as mean  $\pm$  SEM. All data were analyzed using GraphPad Prism software, and statistical significance was defined as  $P < 0.05$ . Two-way repeated measures ANOVA were used to compare time course data between experimental groups, with Sidak's multiple comparisons test to evaluate the effect of diet at each time point. Comparisons between groups obtained at the end of the study were analyzed by T-test. Statistical outliers were identified using the Grubbs' test with  $\alpha = 0.05$ .

## 3. RESULTS

### 3.1 Phenotype characterization of the HFD-induced type 2 diabetes mouse model

**3.1.1 The development of obesity and T2D in mice fed a HFD:** Mice under the HFD regimen ingested 20% less food daily ( $3.28 \pm 0.08$  vs.  $4.17 \pm 0.14$ ;  $P < 0.001$ ) than NFD mice, but daily energy intake was equal due to caloric differences ( $15.56 \pm 0.40$  vs.  $16.60 \pm 0.58$  Kcal/day per mouse,  $P = 0.1483$ ). Body weights were similar at baseline but became significantly higher in HFD mice starting at 4 weeks (Fig. 1A). Both experimental groups continued to gain BW during the course of the study, and after 28 weeks HFD mice showed clear signs of obesity ( $49.1 \pm 1.1$  vs.  $29.1 \pm 0.7$  g;  $P < 0.001$ ). Fasting blood glucose increased significantly by 16 weeks on HFD and remained elevated until the end of the experiment (Fig. 1B). At 28 weeks, the area under curve for ipGTT was higher in HFD than NFD mice ( $P < 0.001$ ; Fig. 1C). HFD mice developed hyperinsulinemia after 8 weeks on diet (Fig. 1D) and IR after 12 weeks as evidenced by a considerable increase in HOMA-IR (Fig. 1E). After 28 weeks on HFD, total cholesterol ( $136.5 \pm 15$  vs.  $62 \pm 8.2$  mg/dL;  $P = 0.003$ ) but not triglycerides ( $47.3 \pm 5.8$  vs.  $38.2 \pm 2.6$  mg/dL;  $P = 0.24$ ) were augmented (Fig. 1F).

**3.1.2 Systolic, diastolic, and mean arterial pressures were elevated in HFD mice:** Systolic and diastolic BP and mean arterial pressure (MAP) values were similar until 18 weeks on the dietary regimen. Systolic BP started to increase in HFD mice but not in NFD mice (Fig. 2A). At 28 weeks, systolic BP in HFD mice reached the highest value compared with baseline ( $135 \pm 7$  vs.  $115 \pm 2$  mmHg;  $P < 0.001$ ) and compared with age matched NFD mice ( $109 \pm 1$  mmHg;  $P < 0.001$ ). Similar effects were observed for diastolic BP ( $103.2 \pm 3.0$  vs.  $89.4 \pm 3.8$  mm Hg;  $P = 0.02$ ; Fig. 2B) and MAP ( $114 \pm 1$  vs.  $99.2 \pm 2.2$ ;  $P = 0.001$ ) at 20 weeks on diet. However, at 24 weeks on diet only MAP continued to increase ( $116 \pm 4.6$  vs.  $100.5 \pm 1.8$ ;  $P = 0.02$ ; Fig. 2C). Heart rate (Fig. 2D) did not change whatsoever during the dietary regimen in any of the mouse groups.

**3.1.3 Albuminuria, Alb/Creat ratio in urine (UACR), and fibrotic markers were not increased in HFD mice:** In NFD mice, albuminuria remained constant during the study (Fig. 3A). Likewise, HFD did not alter the concentration of albumin in urine ( $85.5 \pm 20$  vs.  $79 \pm 6.1$   $\mu$ g/dL;  $P = 0.43$ ) neither UACR ( $21.2 \pm 3.3$  vs.  $22.1 \pm 5.5$   $\mu$ g/mg;  $P = 0.90$ , Fig. 3B) after 28 weeks on diet. The renal expression protein levels of  $\alpha$ -SMA, fibronectin, collagen I and TGF- $\beta$ , did not differ between HFD and NFD (Fig. 3C), suggesting that in this model of HFD-T2D, even at 28 weeks under the dietary regimen, kidney injury was not yet apparent.

### 3.2 Urinary excretion of angiotensinogen (uAGT) was augmented in HFD-T2D mice:

Starting at 20 weeks, uAGT was significantly higher in HFD mice compared to NFD mice. High fat-induced T2D mice exhibited 4-fold higher excretion of uAGT than controls by 28 weeks ( $19.7 \pm 1.4$  vs.  $4.9 \pm 0.8$  ng/day;  $P < 0.001$ ; Fig.4A).

### 3.3 Angiotensin II increased in kidney and urine from HFD-T2D mice:

Ang II levels were elevated in the kidney ( $518 \pm 60$  vs.  $762 \pm 48$  pg/g;  $P = 0.01$ ; Fig.4B) and urine ( $59 \pm 12$  vs.  $131 \pm 20$  pg/day;  $P = 0.02$ ; Fig. 4C) and of HFD mice compared with NFD mice.

### 3.4 Kidney ROS production augmented in HFD-T2D mice:

Renal ROS production was significantly increased in HFD mice compared to NFD mice ( $40 \pm 7$  vs.  $100 \pm 16$  arbitrary units/g;  $P < 0.05$ ; Fig. 5).

## 4. DISCUSSION

In the present study, we demonstrate that C57BL/6 mice fed a HFD for 28 weeks develop obesity and T2D. This mouse model of HFD-induced T2D exhibit body weight increases, hyperglycemia, hyperinsulinemia, insulin resistance, and lipid alterations. Importantly, HFD-induced T2D mice exhibit augmentation of AGT excretion in urine in the absence of albuminuria and renal fibrosis, indicating that uAGT is an early and accurate indicator of intrarenal RAS activation and not a consequence of glomerular damage.

After 28 weeks on HFD, C57BL/6 mice showed clear obesity and T2D phenotypes. Increased body weight gain was detected after 4 weeks, regardless of equal energy intake per day, which is likely due to differences in dietary-induced thermogenesis<sup>23</sup>. This outcome supports the concept that the source of caloric intake is more important than amount. The HFD mice also displayed hyperglycemia, glucose intolerance, insulin resistance, and hypercholesterolemia without significant increases in TG levels<sup>17</sup>.

In this study, emphasis was given to evaluating uAGT as an indicator of intrarenal RAS activation in the absence of overt renal damage. Using a HFD-T2D mouse model, we demonstrated that uAGT increased at 20 weeks and remained elevated until 28 weeks on the diet. The magnitude of increases in uAGT agrees with previous studies using other experimental rodent models<sup>9, 24</sup>. Here, we report lower absolute uAGT values than previously described<sup>25, 26</sup>, which might be attributable to the collection of urine samples after 12 hours of fasting. AGT transcript levels decrease after fasting and are normalized after refeeding, indicating that nutrients, including glucose influence AGT<sup>27</sup>.

Despite augmentation of uAGT excretion, albuminuria was not increased in mice fed a HFD for 28 weeks, even though T2D was established. In contrast, Deji *et al.* show significant increases in albuminuria of C57BL/6 mice on a HFD (60% of Kcal from fat) for 12 weeks indicating diet-induced kidney injury<sup>28</sup>. In the present study, albuminuria did not increase over time. Previous studies report that proteinuria increases with aging in rodents<sup>29</sup> and humans<sup>30</sup>. However, rats fed a normal protein diet exhibit higher UACR than those fed a low protein diet<sup>31</sup>. The lack of difference in the levels of protein expression of kidney fibrotic

factors, including  $\alpha$ -SMA, fibronectin, collagen I, and TGF- $\beta^{21, 32}$  between HFD versus NFD mice, indicate HFD does not stimulate kidney injury in C57BL/6 male mice, at least under the dietary regimen described in the present study. C57BL/6 mice seem to be resistant to the development of glomerulosclerosis, proteinuria, and hypertension as compared to other strains<sup>33</sup>. However, previous studies have shown that C57BL/6 mice may develop alterations, including increases in systolic BP, uAGT, and renal injury in response to either high dietary salt<sup>34</sup>, chronic intermittent hypoxia<sup>35</sup> or during endogenous intrarenal AGT production<sup>36</sup>. The extent to which C57BL/6 mice develop renal alterations upon experimental manipulations may help to explain the existing variability in the renal phenotype to the progression of tissue injury.

Because albuminuria and the protein expression of fibrotic factors in the kidney did not change over time with HFD, even in the presence of a slow progression of the development of T2D phenotype, we suggest that the administration of HFD (45% Kcal from fat) for 28 weeks in C57BL/6 male mice constitute a suitable model of T2D without *a priori* renal damage. The molecular weight of AGT (48–50 KDa) is slightly lower than albumin (66.5 KDa). Thereby, the absence of any sign of apparent glomerular damage in HFD mice argues against the enhancement of uAGT due to increased filtration of systemic AGT<sup>37</sup>. However, since the reabsorption of albumin in the proximal tube significantly impacts the amount that is excreted<sup>38</sup>, we cannot exclude the possibility that filtered albumin is recovered to a greater extent than AGT in the proximal tubule in this mouse model. In the mRen2 rat model, an increase in uAGT is detected without alterations in AGT mRNA or secretion, indicating an extrarenal source<sup>39</sup>. Nevertheless, intratubular AGT delivery to the distal nephron segments provides a substrate for the renin released by the collecting duct, and Ang I and Ang II is subsequently formed in the presence of tubular angiotensin converting enzyme<sup>1</sup>. Indeed, uAGT is considered an index of intrarenal RAS activation in Ang II-infused rats<sup>13, 25</sup>. In the present study, augmentation of uAGT was accompanied by increased Ang II levels in kidney and urine, similar to other rodent experimental models of Ang II-dependent hypertension<sup>13</sup>.

Increased intrarenal AGT contributes to the development and progression of hypertension and kidney injury in different models of experimental hypertension<sup>1</sup>. During T2D, the expression of AGT in renal proximal tubule is stimulated by elevated glucose, which drives the overproduction of ROS<sup>40</sup>. Indeed, treatment with canagliflozin, a sodium glucose transporter inhibitor (SGLT2) inhibits intrarenal oxidative stress in New Zealand Obese T2D mice<sup>41</sup>. Inhibition of SGLT2 may lead to attenuation of ROS generation and subsequent AGT augmentation in PTC. We also found that 28 weeks of the HFD increased ROS production in the kidney. ROS generation occurs in response to hyperglycemia via non-enzymatic glycation of proteins, glucose auto-oxidation<sup>42</sup>, and/or inhibition of anti-oxidative mechanisms through glycosylation of anti-oxidant enzymes<sup>43</sup>. Moreover, in Zucker diabetic fatty rats, ROS increases glomerular AGT expression in a dose- and time-dependent manner by activating extracellular-regulated kinase (ERK)/JNK pathway<sup>24</sup>. Although in the present study, we did not measure the time dependent changes of intrarenal Ang II, ROS, collagen, or fibronectin; collected evidence supports that the increases in uAGT in mice subjected to long-term HFD is mediated, at least in part, via increased ROS generation in response to hyperglycemia<sup>40</sup>, since both appeared in the absence of overt renal injury.

In the present study, the elevation in systolic BP was concomitant with the augmentation of uAGT, indicating that increased uAGT could contribute to the regulation of BP. Studies in transgenic mice support the concept that kidney-specific enhancement of the RAS contributes to BP regulation<sup>44</sup>. Sachetelli *et al.* reported that renal overproduction of AGT increases systolic BP in mice<sup>45</sup>. Proximal tubule-derived AGT regulates BP through luminal Ang II generation<sup>1, 46</sup> which stimulates Na<sup>+</sup> reabsorption by increasing proximal sodium/hydrogen exchanger 3 activity<sup>47</sup> and distal epithelial sodium channels<sup>48, 49</sup>. Moreover, in the presence of ROS, the AGT molecule undergoes a subtle conformational change and releases Ang I more effectively when exposed to renin, thus facilitating Ang II generation<sup>50</sup>.

One caveat of the current study is that we did not continue the study past 28 weeks of the HFD to observe overt markers of renal damage. The use of a diet with lower fat content was intentional, to avoid quick induction of renal injury and allow us to discern whether increased uAGT occurs in response to intrarenal RAS activation or glomerular damage. By examining RAS expression in kidney tissues along with ROS, we were able to show that uAGT appears in the absence of other markers of renal injury. Additional experiments should compare this novel biomarker with other clinical assays for oxidative stress including urinary 8-Isoprostane and TGF-beta. These experiments would determine whether uAGT increases are detected before other accepted markers for oxidative stress.

In conclusion, the data indicate that uAGT is an early marker of intrarenal RAS activation in mice with HFD-induced T2D, in the absence of signs of kidney injury such as albuminuria. Our data support a recent clinical study showing that in T2D patients, increases in uAGT precede the onset of albuminuria<sup>51</sup>. Increased uAGT excretion could be mediated, at least in part, by augmented kidney ROS production. Type 2 diabetes is a long-term disease and renal injury is an inevitable complication. In perspective, our results suggest that clinical evaluation emphasis should be placed on the detection of increased urinary excretion of biomarkers as early indicators of intrarenal RAS activation, rather than renal injury markers only, which are the consequence of the disease but not the cause. The complex interactions between the intrarenal RAS and the development of renal injury associated with T2D induced by HFD is critical to optimize current therapeutic strategies to improve precision medicine.

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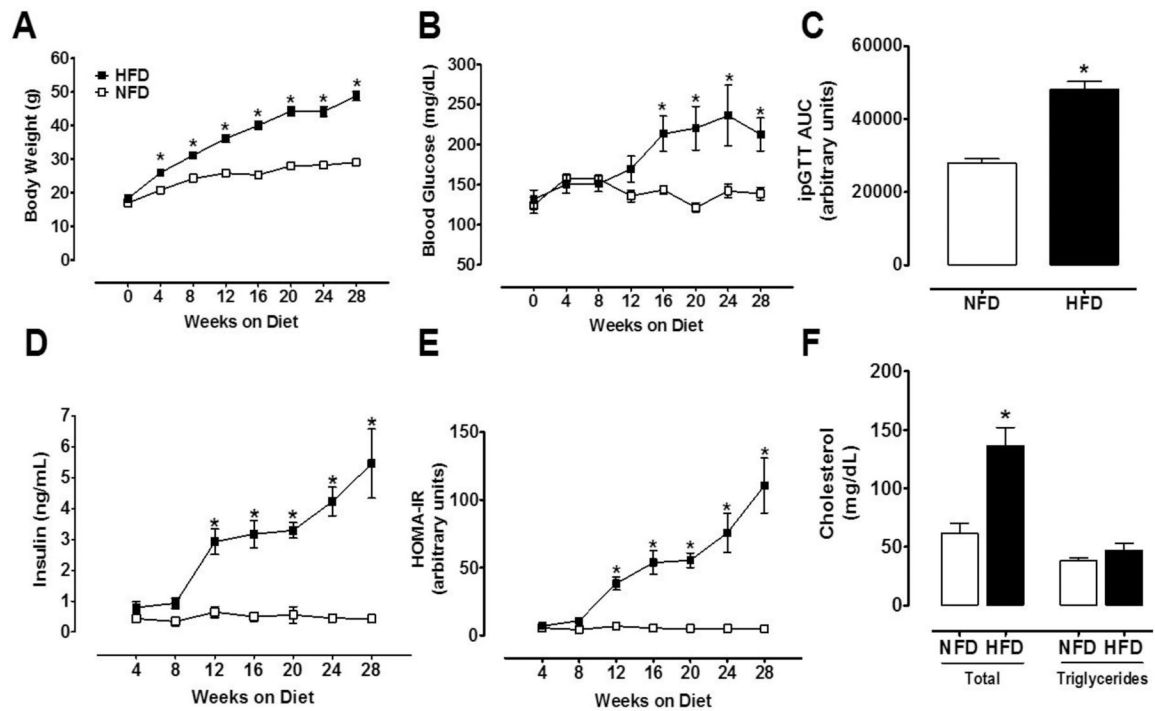
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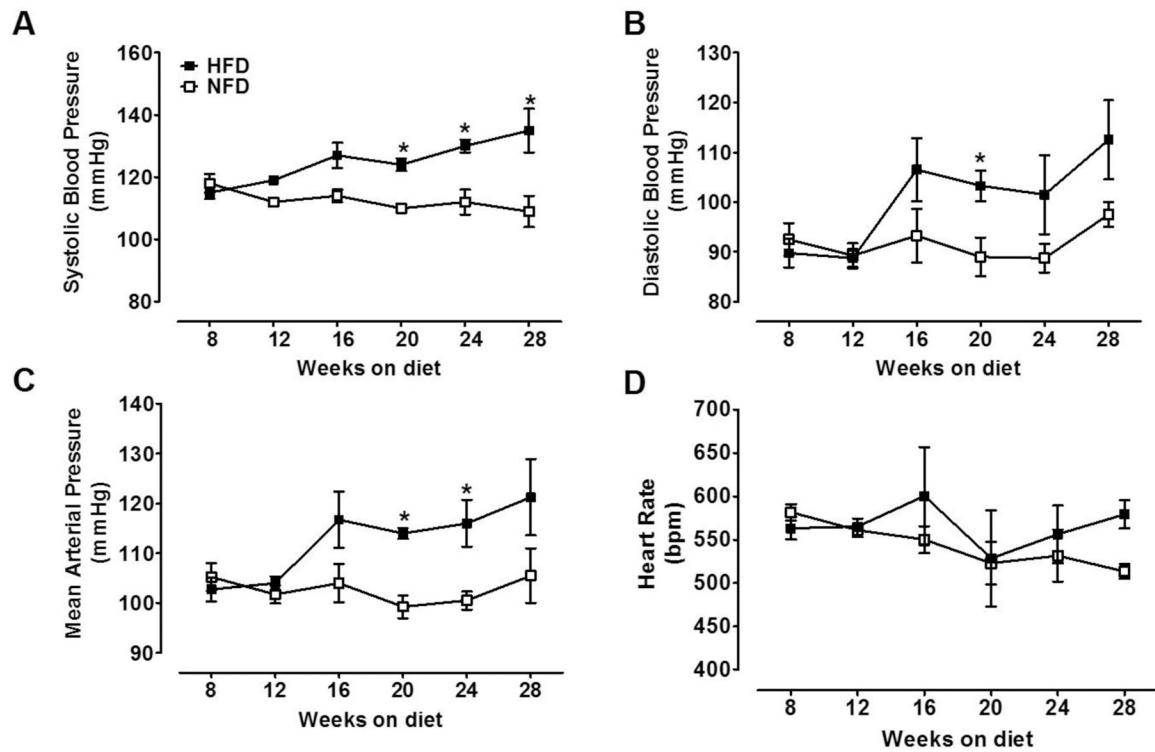
### Highlights

- Whether the augmented excretion urinary AGT (uAGT), locally generated in the proximal tubule of the kidney, is an indicator of glomerular damage rather than intrarenal RAS activation, remains unclear.
- Using a mouse model of slow progression of T2D phenotype induced by high fat diet (HFD), we demonstrated that augmentation uAGT occurred in the absence of overt albuminuria. These changes were accompanied by intrarenal increases in ROS and Ang II concentrations.
- In HFD-induced T2D mice, increased uAGT precedes glomerular damage and kidney tissue injury and indicates intrarenal RAS activation.



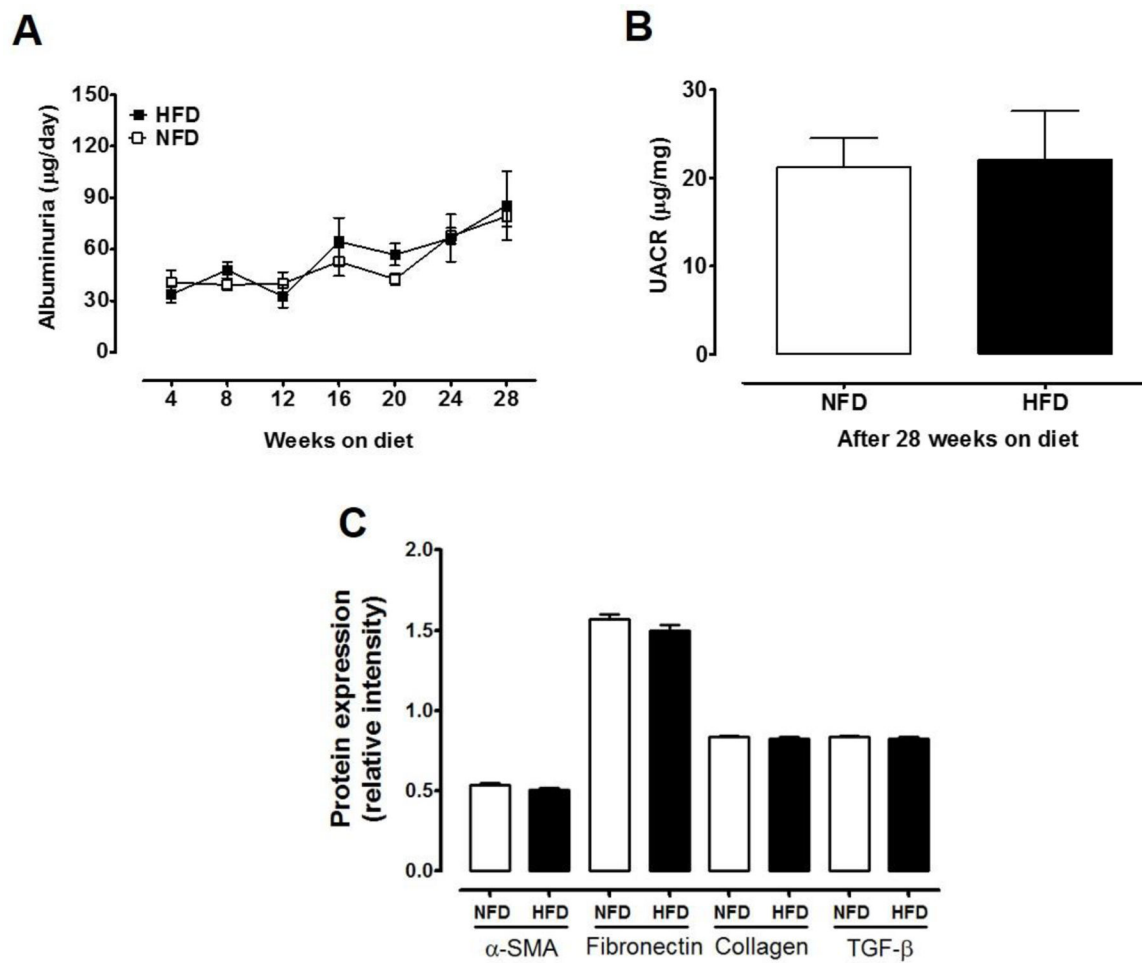
**Figure 1. Metabolic phenotype of mice fed a HFD.**

After 28 weeks, HFD-fed mice showed marked signs of obesity and T2D as evidenced by (A) Body weight (2-way ANOVA, diet effect:  $P < 0.001$ ), (B) Blood glucose (2-way ANOVA, diet effect:  $P < 0.05$ ), (C) ipGTT (t-test,  $P < 0.001$ ), (D) Plasma insulin (2-way ANOVA, diet effect:  $P < 0.001$ ), (E) HOMA-IR (2-way ANOVA, diet effect:  $P < 0.001$ ), and (F) Plasma total cholesterol (t test  $P < 0.003$ ). NFD, normal fat diet (white); HFD, high fat diet (black); Data are mean  $\pm$  SEM.

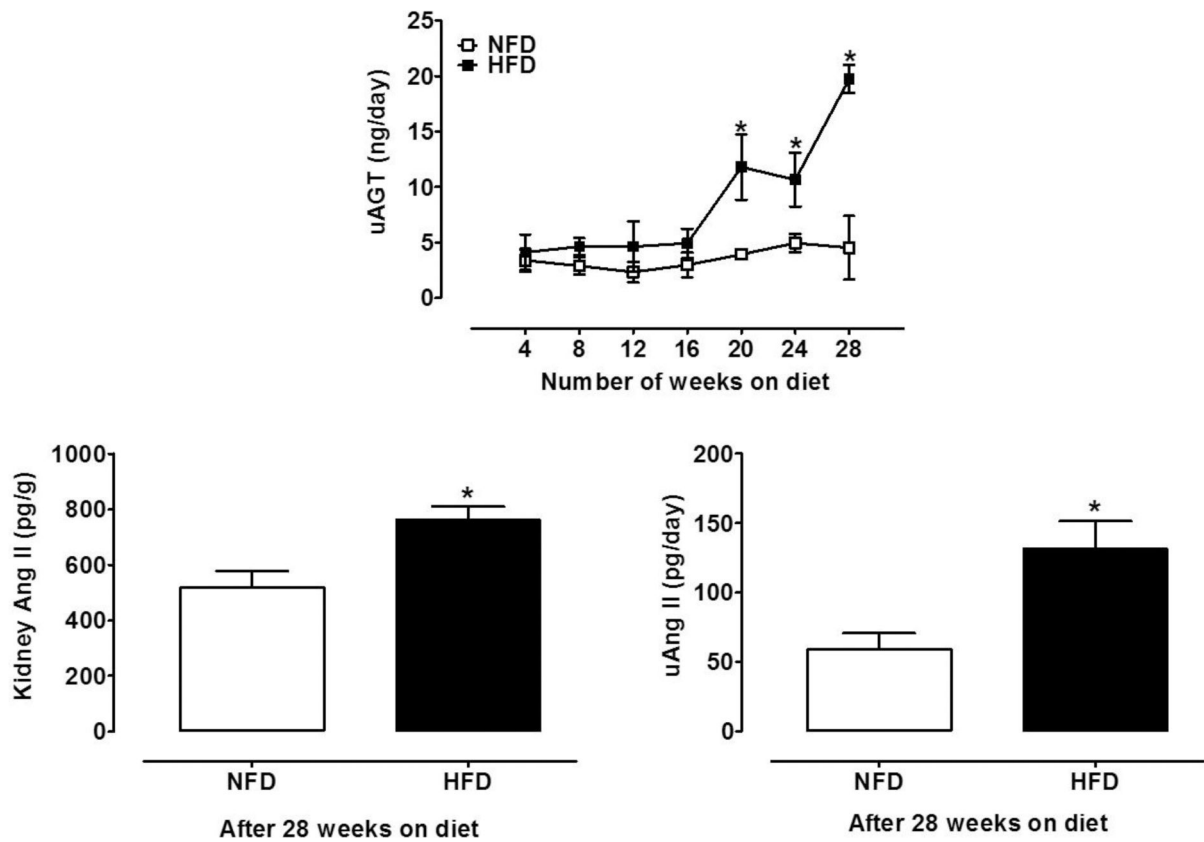


**Figure 2. Systolic BP in HFD-induced T2D mice and NFD mice.**

Radiotelemetry measurements are representative of 24 h recordings. **(A)** Systolic BP was higher than in age-matched NFD mice after 20 weeks on the diet (2-way ANOVA, diet effect:  $P < 0.001$ ). **(B)** Diastolic BP was higher among the groups at 20 weeks (2-way ANOVA, diet effect;  $P < 0.04$ ). Mean arterial pressure (MAP) was elevated at 20 and 24 weeks (2-way ANOVA, diet effect;  $P < 0.001$ ). **(D)** Heart rate did not change between experimental groups. NFD, normal fat diet (white); HFD, high fat diet (black); Data are mean  $\pm$  SEM.



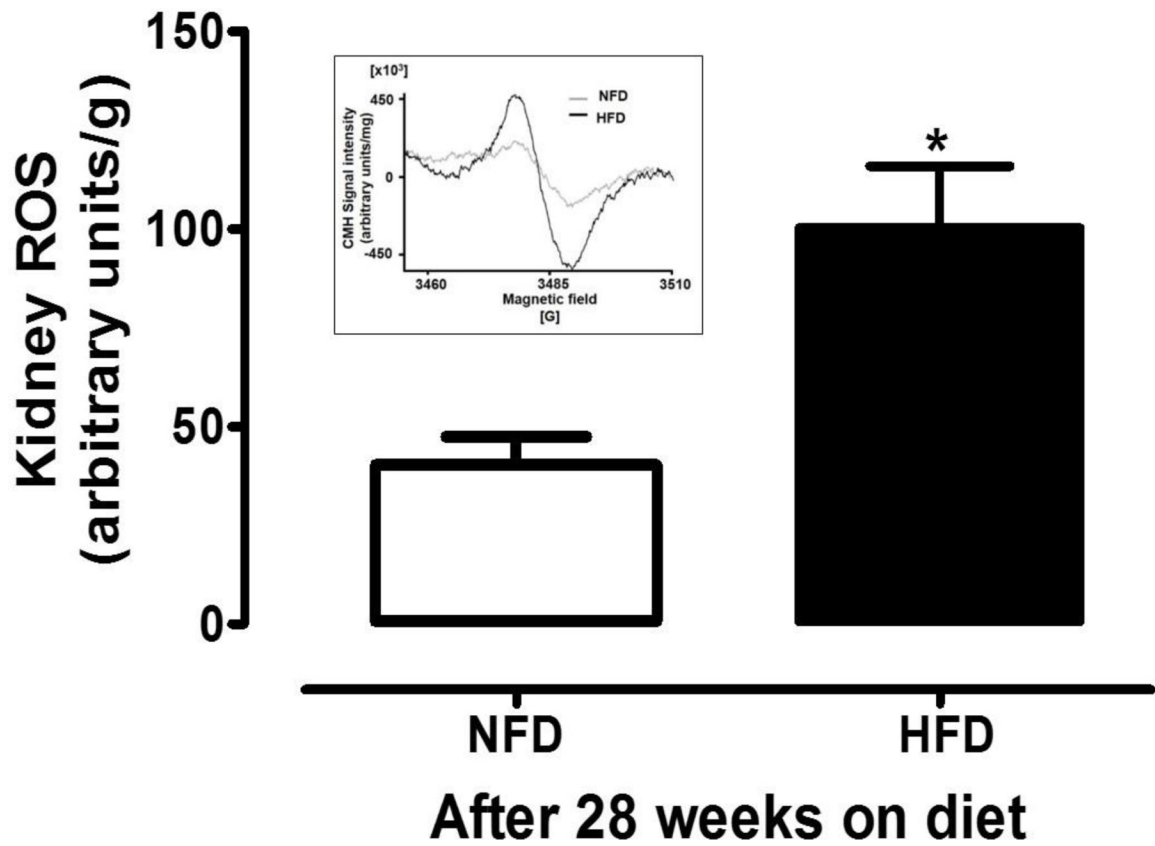
**Figure 3. Albuminuria and UACR in HFD-induced T2D mice and NFD mice.** (A) Albuminuria was not impacted by diet (2-way ANOVA, diet effect:  $P=0.43$ ) but similarly increased in both groups over time (time effect:  $P<0.0001$ ; Sidak's test of week 28 vs. week 4:  $P<0.05$ ). (B) UACR was not different after 28 weeks on diet (t-test,  $P=0.90$ ). (C) Expression levels of TGF- $\beta$ , fibronectin, collagen I, and  $\alpha$ -SMA measured by Western blot in whole kidney samples were not different between experimental groups (t-test,  $P>0.05$ ). NFD, normal fat diet (white); HFD, high fat diet (black); Data are mean  $\pm$  SEM.



**Figure 4. Urinary AGT, and kidney and urine Ang II levels in the HFD-induced T2D mouse model.**

After 28 weeks on the diet, HFD mice showed a significant elevation in (A) urinary excretion rate of uAGT (2-way ANOVA, diet effect:  $P < 0.001$ ; time effect:  $P < 0.001$ ; Sidak's test of week 28 vs. week  $P < 0.001$ ). In addition, HFD increased (B) kidney Ang II levels (t-test,  $P = 0.01$ ) and (C) urine Ang II (t-test,  $P = 0.02$ ). NFD, normal fat diet (white); HFD, high fat diet (black); Data are mean  $\pm$  SEM.





**Figure 5. Kidney ROS production measured by ESR spectroscopy in whole kidneys from NFD and HFD mice.**

Characteristic ESR spectra of CMH representing samples from HFD and NFD fed animals were shown in upper left insert and the cumulative data represented in the bar graphs. The HFD-T2D mice showed 2.5-fold elevation in ROS production compared to NFD mice. ROS production was normalized to the dry weight of the corresponding kidney samples in grams. NFD: normal fat diet (white); HFD: high fat diet (black); ROS: reactive oxygen species; ESR: electron spin resonance. Data are mean  $\pm$  SEM.