Effects of high fat diet and losartan on renal cortical blood flow using contrast ultrasound imaging

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RUNNING HEAD: Obesity-related kidney injury and contrast ultrasound imaging

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ABSTRACT

Obesity-related kidney disease occurs as a result of complex interactions between metabolic and hemodynamic effects. Changes in microvascular perfusion may play a major role in kidney disease; however, these changes are difficult to assess in vivo. Here, we used perfusion ultrasound imaging to evaluate cortical blood flow in a mouse model of high fat induced kidney disease. C57BL/6J mice were randomized to a standard diet (STD) or a high-fat diet (HFD), for 30 weeks, then treated either with Losartan or a placebo for an additional 6 weeks. Non-invasive ultrasound perfusion imaging of the kidney was performed during infusion of a microbubble contrast agent. Blood flow within the microvasculature of the renal cortex and medulla was derived from imaging data. An increase in the time required to achieve full cortical perfusion was observed for HFD mice relative to STD. This was reversed following treatment with losartan. These data were concurrent with an increased glomerular filtration rate in HFD mice compared to STD or HFD-losartan-treated mice. Losartan treatment also abrogated fibro-inflammatory disease, assessed by markers at the protein and messenger level. Finally, a reduction in capillary density was found in HFD mice, and this was reversed upon Losartan treatment. This suggests that alterations in vascular density may be responsible for the elevated perfusion time observed by imaging. These data demonstrate that ultrasound contrast imaging is a robust and sensitive method for evaluating changes in renal microvascular perfusion, and that cortical perfusion time may be a useful parameter for evaluating obesity-related renal disease.

KEYWORDS: ultrasound contrast imaging, obesity-related kidney disease, renal perfusion
INTRODUCTION

The growing epidemic of obesity is a serious health and economic burden throughout the world. The consequence of obesity over time is characterized by insulin resistance, hyperglycemia, atherosclerosis, dyslipidemia and hypertension. This cluster of risk factors referred to as metabolic syndrome occurs with 50 % prevalence in patients 50-60 years-old (4), and contributes to cardiovascular disease. Obesity is a major risk factor for diabetes and hypertension which together account for about 70 % of all cases of end-stage renal disease (6). However, there is growing evidence suggesting that obesity by itself may be an independent risk factor for the development of vascular dysfunction and renal disease (21, 44, 66). Obesity-related kidney disease occurs as a result of complex interactions between metabolic and hemodynamic factors and is characterized by changes of renal perfusion, vascular dysfunction, as well as albuminuria, glomerulosclerosis and tubulointerstitial fibrosis (20, 37, 48, 50). Alterations in blood flow within the cortex or the medulla may therefore be likely to occur in obesity-related kidney disease. However, techniques for directly measuring perfusion of the renal microvasculature, either in small animal models or in clinical medicine, are limited.

Ultrasound perfusion imaging using non-targeted vascular contrast agents provides a real-time and noninvasive method to assess microvascular perfusion in living subjects (7). Contrast agents for ultrasound perfusion imaging are gas-encapsulated microbubbles stabilized by a shell of lipid, protein, or polymer (8). Microbubble contrast agents are generally between 1 – 8 μm in diameter, allowing uninterrupted passage through capillaries. Previous studies have demonstrated that the rheology of microbubble agents is similar to that of erythrocytes (22, 23, 34) and behave as purely intravascular flow tracers. Unlike iodinated contrast agents and chelates used for MRI, microbubble agents are not filtered by the kidney. Thus, the contrast images obtained using this technique represent true vascular perfusion.
Several microbubble-based imaging agents have been approved for clinical cardiac and radiological indications in the US, Europe, China, and elsewhere. Clinical applications in the kidney are generally limited to characterization of overt vascular anomalies (such as stenosis and segmental infarction) and qualitative evaluation of perfusion abnormalities. Here, we sought to test whether this technique has the sensitivity to detect subtle changes in renal cortical blood flow that may occur in the context of glomerular fibrosis, and subsequent therapy, in a model of obesity-related kidney disease.

MATERIALS AND METHODS

Animals and interventional study. Male C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME). All animal procedures were approved by the Institutional Animal Care and Use Committee of University of California, San Diego. Starting at six weeks of age, mice were fed a high-fat diet (HFD, n=12) in which 60% of total calories were from fat, 20% from protein, and 20% from carbohydrate (Research Diets, New Brunswick, NJ) or standard diet (STD, n=7), consisting of 5% fat and 24.5% protein for 30 weeks. Mice in the HFD group were further randomized into two groups of six. The first group (n=8) was treated with Losartan (Sigma), an angiotensin II receptor 1 antagonist, at 5 mg/kg/day in drinking water for 6 weeks starting after 30 weeks on HFD. The second group (n=6) served as a placebo control. It has been previously demonstrated that there is not a significant antihypertensive effects caused by this dose of losartan (5). The concentration of compounds was adjusted once every other day based on water consumption and B.W. The food intake as well as the body temperature was also measured.

All mice were imaged at week 36, and then sacrificed. Mice were placed in metabolic cages for 24-h urine collection before treatment and on the last day of the treatment period. After euthanasia, blood samples were collected and portions of both kidneys were snap-frozen in
liquid nitrogen for RNA isolation. An additional portion was frozen in OCT or fixed in 4% PAF for immunostaining.

Ultrasound contrast agent. This study was performed using a perfusion ultrasound contrast agent (Targetstar® P; Targeson Inc) commercially available for life science research. This agent is a lipid encapsulated perfluorocarbon microbubble incorporating a coating of poly(ethylene glycol). The mean diameter of the microbubble particles is 2.2 um.

Ultrasound Imaging. All ultrasound imaging was performed on a Siemens Sequoia 512 (Siemens Medical Solutions), using a high-frequency clinical transducer (15L-8). All contrast images were acquired at a dynamic range of 70 dB, mechanical index of 0.19 – 0.24, and gain of 0 dB. No persistence or edge enhancement was applied.

On the final study day, mice were anesthetized with ketamine (100mg/kg) and xylazine (10mg/kg) and placed in the left lateral decubitus position on a heated stage. The skin along the flank was first shaved with an electric razor, then depilated with a commercial crème product. A cannula consisting of a 10 cm length of PE-10 tubing connected to a 27G needle bent into an L shape was inserted into the retro-orbital sinus and secured to the stage with tape. A mound of heated ultrasound coupling gel was placed on the right flank, and the ultrasound transducer was fixed in place with a flexible clamp approximately 1 inch above the skin surface. Images of the right kidney were first acquired in B-mode at a center frequency of 14 MHz, and the transducer position was adjusted so as to obtain a uniform view of the kidney in long axis. The same field of view was then acquired in contrast imaging mode (Cadence® CPS), and the contrast gain was adjusted such that the noise was just slightly visible. Contrast agents were diluted 1:10 by volume in sterile 0.9% saline and infused at a rate of 60µl/min using a syringe pump (Harvard Apparatus). Entry of the contrast agent into
the kidney was apparent on ultrasound imaging within several seconds of beginning the
infusion. After a one minute period of infusion to allow the vascular concentration of contrast
agent to reach steady state, a high mechanical index burst was applied (MI=0.7, duration
2.0s). This disrupted all contrast agents within the field of view, creating a “negative bolus”
effect. A clip consisting of ~2 seconds before the burst and 20 seconds after was digitally
recorded at the maximum frame rate possible (14-18 Hz). Complete re-perfusion of all
segments of the kidney was found to reproducibly occur within 4-6 seconds of the destructive
burst.

Ultrasound Image Analysis. Data analysis was performed in Image J (v 1.46r, National
Institutes of Health). The first 20 seconds of digital video after the burst were analyzed for
each animal. Polygonal regions of interest encompassing the renal medulla or cortex were
defined, being careful to avoid the interlobular arteries. Ultrasound imaging data is typically
presented with color mapping (here, in shades of orange) and compression in order to
highlight relevant aspects of the image during the scan; this can confound quantitative
analysis of imaging data by the introduction of non-linearity in the relationship between the
image intensity and the true microbubble concentration. Therefore, the nonlinear color map
and post-processing settings were reversed to yield linearized intensity data. The linearized
intensity data were baseline-subtracted and normalized by peak intensity. Time-to-peak
(measured in seconds) was defined as the time required for the normalized intensity to reach
95% of the peak intensity. This was computed from a single time-intensity curve for each
animal.

Glomerular Filtration Rate. Twenty minutes after the infusion of ultrasound contrast agents
was stopped, FITC-inulin was injected in bolus in order to measure the glomerular filtration
rate (GFR) based on published methods (12, 47, 56, 62). 5% inulin was dissolved in normal saline and dialyzed 24 hours to remove unbound inulin. This solution was sterile filtered, and then injected (2μl/g/b.w.) retro-orbitally to anesthetized mice. Twenty microliters of blood were collected from the tail vein at 3, 5, 7, 10, 15, 30, 60 and 75 minutes into a Na⁺-heparinized microcap. The samples were spun down and 1μl of plasma was reconstituted in HEPES buffer. The fluorescence was then determined using a Nanodrop ND-3300 fluorospectrometer (Nanodrop Technologies, USA). GFR was calculated using a 2 phase exponential decay principle using Graph Pad (Prism 5) software.

Urine and plasma analysis. The urine albumin and creatinine were measured with a mouse Albuwell ELISA kit and a Creatinine Companion kit (Exocell, USA). As an index of oxidative stress, urine samples were also analyzed for hydrogen peroxide by Amplex red assay (Invitrogen, USA) following the manufacturer’s protocol.

Quantitative real-time PCR. Frozen kidneys (-80°C) samples were homogenized and total RNA was then extracted. The mRNA quantification was performed using a 2-step real-time reverse-transcriptase polymerase chain reaction. Real-time PCR was performed on kidney using the primers for angiotensinogen, renin, MCP-1, α1-type I collagen, α1-Type IV collagen, and β-actin as a housekeeping gene. Relative gene expressions were calculated using the $2^{-ΔΔCT}$ method.

Immunohistochemistry. Immunostainings of macrophages and endothelial cells on paraffin-embedded mouse kidneys were performed using rat anti-CD43 antibody (BD Biosciences) and rat anti-mouse CD31 primary antibody (AbD Serotec), respectively. Quantitation of CD43–positive cells was evaluated by a semi-quantitative analysis as described previously.
Briefly, the distribution of positive cells in the different histological structures of the renal tissue was performed on one section per experimental animal. For each section, 10 square fields (0.084 mm²/field) were observed at x400 magnification. The relative area occupied by CD31 in renal tissue was evaluated by a computer-assisted morphometric approach. Images were obtained with a DeltaPix digital camera (model DP200) and further analysed using the National Institute of Health Image J software (NIH, Bethesda, MD) to quantify the percentage of stained area. The sections were analysed by scanning 10 fields at x200 magnification.

Statistical analysis. Results are presented as mean values ± SEM. The level for statistical significance was defined as p<0.05. Analyses were carried out using Graph Pad Prism Software version 4.03. Difference between data groups were evaluated for significance using, 1-way ANOVA and Newman-Keuls post-hoc test for multiple comparisons.

RESULTS

Model Characterization and Metabolic Data

Metabolic data of mice fed a normal or high fat diet with or without Losartan treatment are shown in Figure 1. The temporal evolution of the body weight presented in Figure 1A showed a significant increase in all groups from T0, corresponding to the day 0, to T4 which corresponds to the end of the experimental protocol (Week 36). However, the body weights of mice fed a HFD or HFD+Losartan were significantly higher than those of STD mice. At Week 36, the body weight reached 58.53 ± 1.08 g (P<0.001) in HFD group, 58.18 ± 0.78 g (P<0.001) in the HFD+Losartan group and 31.86 ± 0.74 g in STD mice. Losartan treatment did not prevent body weight gain. Kidney weight of HFD mice were significantly higher than these of STD mice (0.0246 ± 0.0007 g/mm of tibia length versus 0.0171 ± 0.0003 g/mm of...
Kidney hypertrophy was significantly attenuated with Losartan compared to HFD mice treated with placebo (0.0210 ± 0.0006 g/mm of tibia length versus 0.0246 ± 0.0007 g/mm of tibia length, respectively; P=0.001). Nevertheless, the kidney weight was still higher compared to STD mice (P<0.001). Blood glucose was significantly higher in HFD mice throughout the experimental protocol (T0 to T4). Moreover, at week 36, the level of blood glucose was 171.2 ± 8.5 mg/dl (P=0.045) compared to 122.6 ± 3.8 for the STD mice. Treatment with losartan tended to reduce the blood glucose although results were not significant as compared to HFD alone.

As illustrated in Figure 2, the GFR was significantly higher in mice fed a HFD (252.2 ± 24.8 µl/min; P=0.0043) compared to the mice fed a STD (157.7 ± 11.2 µl/min). Moreover, increased GFR was significantly attenuated by Losartan treatment (183.4 ± 21.5 µl/min; P<0.05) (Figure 2A). In addition, high fat diet induced a significant elevation of albuminuria as measured at week 30 (82.23 ± 9.45 µg/mg Cre HFD-before placebo (P=0.021) and 81.16 ± 16.13 µg/mg Cre HFD-before Losartan (P=0.014) versus 35.14 ± 5.22 µg/mg Cre STD-before placebo) which was further enhanced at week 36 in HFD mice receiving the placebo (107.20 ± 10.77 µg/mg Cre; P=0.002). Interestingly, HFD mice treated with Losartan did not present a further increase in albuminuria (83.04 ± 11.65 µg/mg Cre), showing a beneficial effect of Losartan on renal function (Figure 2B).

Perfusion Imaging

As it has been demonstrated elsewhere (41, 68), microbubble contrast agents exhibit a triphasic enhancement pattern within the intact kidney. Enhancement of the renal artery and interlobular arteries is visualized first, followed by enhancement of the renal cortex, and then of the renal medulla. Each of the three phases was detectable in all mice with the imaging
system described here (Figure 3A-STD, B-HFD and C-HFD+Losartan). Out of 21 kidneys imaged, data from one scan was discarded due to excessive imaging artifact in the signal. In mice fed a standard diet (STD), time-intensity analysis showed a very rapid entry of contrast into the cortex, while the medulla took several seconds longer to fully perfuse. This was appreciable from the time-intensity curves (Figure 4A). In HFD mice, the time-intensity curve for the cortex manifested a rightward shift, representing a longer duration of time required for the cortex to fully perfuse (Figure 4B). Finally, the third row illustrating the sequence taken from a HFD treated with Losartan revealed that Losartan alleviated the changes observed in the cortical perfusion in HFD (Figure 4C). The delay observed in the HFD mice was quantified in the time-to-peak (TTP) calculation, and a significant increase in TTP was found for HFD mice relative to STD (Figure 4F). The observed delay in cortical perfusion was reversed in HFD mice treated with losartan (Figure 4C), and the TTP for these mice was not statistically different to that of STD mice (Figure 4F). No change in TTP among cohorts was found for the medulla (Figure 4G).

There was no difference in the baseline or peak enhancement among any of the cohorts (Figure 4D, E), suggesting that the total volume of contrast agent entering the kidneys was similar.

Microvascular Density

Immunostaining of CD31, a marker of endothelial cells, was performed on paraffin-embedded kidney sections in mice fed a STD, a HFD or a HFD-Losartan (Figure 5A-D). The density of cortical microvessels, assessed by CD31, was reduced in the high fat diet cohort. Treatment with Losartan mediated a partial recovery (Figure 5A-D), although vessel density was still slightly less than that of mice on STD.
Determination of inflammatory and pro-fibrotic markers

The data in table 1 illustrates the mRNA level of several markers involved in renin-angiotensin pathway, fibrosis, and inflammation. As observed, the angiotensinogen and renin mRNA level did not differ between groups. Collagen Type I and IV mRNAs were elevated with HFD, and reduced to baseline levels with losartan treatment. Monocyte Chemotacttractant Protein-1 (MCP-1), a specific marker of inflammation, showed a significant higher mRNA level in mice fed a HFD. The increase in MCP1 was clearly attenuated by Losartan (Table1). Moreover, the significant increase of macrophages (CD43-positive cells) with HFD was significantly attenuated with Losartan (Figure 6A). Finally, the urinary hydrogen peroxide level, also considered as a marker of renal inflammation was significantly higher after HFD and was reduced with Losartan (Figure 6B).

DISCUSSION

This study demonstrates the use of perfusion ultrasound imaging as a fast and practical technique to measure regional kidney blood flow in mice. Here, C57bl/6 male mice were fed a HFD. Feeding HFD to mice is known to induce metabolic alterations (10), including increased body weight, kidney hypertrophy, hyperglycemia (as observed in Figure 1). A continuous infusion of MB-contrast agent was administered intravenously to anesthetized mice fed a STD or a HFD. In addition, to better demonstrate the use of US-contrast agents in monitoring modulations in renal blood perfusion, Losartan was administered with HFD concurrently for 6 weeks. Losartan is a specific angiotensin II receptor 1 antagonist. Angiotensin II is a crucial mediator in the progression of obesity and diabetes related kidney disease (1, 33, 60, 61). Angiotensin II participates to the hyperfiltration and glomerulosclerosis through hemodynamic and non-hemodynamic effects (16, 35, 39, 40, 43, 45).
The use of non-targeted microbubble contrast agent in a mouse model of obesity-related kidney disease demonstrated that in mice fed a standard diet the cortex was observed to fully perfuse rapidly, with perfusion of the medulla occurring several seconds later. A significant delay in the cortical perfusion time was observed in mice on high fat diet, and this behavior was quantified using the time-to-peak intensity calculation. The increase in cortical time-to-peak was abrogated upon treatment with losartan. Similar differences between cohorts were found for inflammatory markers at the protein and messenger level. Additionally, a rarefaction in cortical capillaries was found in HFD-treated mice, and reversed with losartan, suggesting that alterations in blood vessel density may be responsible for our imaging findings.

In order to link the imaging data to pathological features of obesity-related kidney disease, GFR and albuminuria were determined. We found that HFD significantly increased GFR and albuminuria; this increase was abrogated by losartan treatment. These data might result from the vasodilation of renal afferent arteriole and vasoconstriction of efferent arteriole in response to circulating vasoactive substance. This leads to an increase of the hydrostatic pressure in the glomerular capillaries which contributes to an elevated GFR and albuminuria. However, it has been shown that ANG II can exaggerate the vasoconstriction response of the efferent arteriole in obese rats, favoring glomerular dysfunction (49). In our study, since Losartan, an AT1 angiotensin receptor antagonist, prevents the increased GFR and restores the cortical blood flow, we might hypothesize that ANG II acts through the AT1 receptor which has been demonstrated to be distributed on renal efferent arteriole (17, 29).

The delay in the cortical perfusion time observed in mice on high fat diet might result from the interplay between hemodynamic and non-hemodynamic mechanisms. It is known that impaired vascular regulation is associated with inflammatory and fibrotic cytokines as well as albuminuria, and may result in glomerular hyperfiltration (53, 70). In our study, HFD mice
showed an increase in inflammatory (MCP1 mRNA level, hydrogen peroxide and CD43+ macrophages) and fibrotic (Collagen IV mRNA level) markers while Losartan treatment reversed this. Therefore, the partial resolution to the pro-inflammatory state in losartan-treated HFD mice can be related to the prevention of the cortical perfusion. It has been shown that angiotensin receptor antagonist has a beneficial effect on endothelial dysfunction as well as in insulin resistance (15, 25, 65). Moreover, a recent study demonstrated that losartan treatment could restore the AMPK activation in the kidney in a similar HFD model (11). AMPK is considered as a cellular energy sensor which plays a crucial role in glucose metabolism and has been demonstrated to reduce renal inflammation with HFD feeding (3, 10, 38, 52).

Finally, we assessed the peritubular capillary density by CD31 expression. Our data indicated a loss of peritubular capillaries with HFD, and partial resolution upon losartan treatment. This data suggests that changes in vascular density may be responsible for our imaging observations.

There is a paucity of quantitative methods for non-invasively assessing renal perfusion. In the clinical context, CT has been the preferred modality for renal imaging, although recent concerns about toxicity of some CT contrast agents have largely diminished its use in patients with renal disease. Contrast MRI has shown utility, although the cost, relatively low throughput, and concerns about contrast agent nephrotoxicity have limited its use in clinical and research settings. Doppler ultrasound imaging is able to depict blood flow in large vessels, but generally does not have the sensitivity to depict flow at the microvascular level. In small animals, the gold standard for imaging perfusion remains laser Doppler flowmetry. This technique provides accurate measurement of erythrocyte flux, although has limited depth penetration and requires surgical manipulation for use in the kidney. The use of microbubble contrast agents with a non-destructive contrast imaging mode (such as that implemented on most modern ultrasound scanners) enables the non-invasive use of ultrasound
imaging for detection of blood flow at the capillary level. This technique can be readily scaled for use in small animals by selection of a high frequency probe, does not require any surgical procedures, and can be accomplished in a matter of minutes.

Animal studies have demonstrated a strong correlation between blood flow derived from perfusion ultrasound imaging and flow probes (19, 31, 68) and radiolabelled microspheres (58) with various microbubble formulations, suggesting that perfusion ultrasound accurately reflects blood flow. Subsequent studies have been performed in rat (13, 31, 41), rabbit (59), dog (63, 64, 68), swine (24, 69), mouse (55, 57) and in isolated organs (54). Contrast ultrasound imaging of the kidney is not in widespread clinical use, although studies have demonstrated potential utility for evaluation of focal abnormalities (46) and in identifying rejection following renal transplant (14, 27, 28). Of particular note, Kleinert (2012) (30) showed that cortical TTP correlated to GFR in a small study of scleroderma patients, and Kalantarinia (2009) (26) demonstrated an increase cortical blood velocity in healthy subjects following a high-protein meal. These collective data demonstrate the perfusion ultrasound as an accurate, reproducible, and translatable method for non-invasively assessing renal blood flow.

Much of the work in validating perfusion ultrasound imaging in the kidney has been performed using surgically (19, 58, 68) or pharmacologically (13, 31, 41, 57) induced changes in renal blood flow, but not in models of common clinically relevant diseases. To our knowledge, the data presented here is the first evidence that this technique can detect chronic kidney disease and its response to treatment. Moreover, the relatively simple procedure utilized here provided sufficient robustness without the need for extensive image processing or analysis. This may be of benefit to researchers utilizing small animal models, and also as a clinical tool for routine evaluation of renal perfusion in patients with metabolic disease.
Our study has several limitations. Kidneys were evaluated in only one imaging plane, which may introduce variability due to undersampling (13). Volumetric imaging may therefore be required to achieve the greatest sensitivity, although at the expense of increased procedure time and complexity. Imaging in our study was performed using a clinical ultrasound scanner operating at 14 MHz, offering a spatial resolution of ~250 μm. Ultra-high frequency ultrasound, operating at 40 MHz and with a spatial resolution of ~100 um, can be used with microbubble contrast agents (51). However, as noted by Sullivan (2009), the sensitivity to contrast agents using ultra-high ultrasound scanners is generally limited (57). We found that use of a clinical scanner, operating at an imaging frequency close to the microbubble’s natural resonant frequency, offered a suitable balance between spatial resolution and contrast sensitivity for imaging the murine kidney. Finally, we used a relatively simple analysis method based on time-to-peak intensity. Previous studies have extracted quantitative parameters relating to blood volume and velocity based on curve fitting of time-intensity data. The most common analysis utilizes a shifted exponential function (26, 31, 57, 67, 68). This method relies upon the a priori assumption that the time intensity curve follows the mathematical model used, an assumption about which there is some disagreement in the field (2, 18, 32, 36, 42). To our surprise, we found that our simple time-to-peak analysis, which requires no assumptions regarding the mathematical shape of the time-intensity curve, offered sufficient robustness to detect changes in cortical blood flow in our animal model.

In summary, our study demonstrates that the kidney responds to the challenge of high fat feeding, exhibiting renal function impairment, inflammation, and fibrotic response at the gene expression, histologic and functional level. Losartan treatment improved the overall kidney function status by preventing kidney hypertrophy, reducing renal inflammation, preventing hyperfiltration and preventing further increased albuminuria. In our study, Losartan was also used to validate the use of US-contrast agents in monitoring modulations
in renal blood perfusion. Hence, US-contrast agent technique was proven to be sensitive enough to monitor the prevention of the cortical perfusion in losartan-treated HFD mice. Therefore, we demonstrate that the use of perfusion ultrasound imaging may be an useful tool for non-invasively assessing the extent, and monitoring the resolution, of obesity-related kidney disease.

Acknowledgments

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Disclosures

JJR and DJS are employees and own stock in Targeson, Inc.


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**FIGURE CAPTIONS**

**Figure 1.** Temporal evolution of body weight (A) in mice fed a STD, a HFD or HFD-Losartan. Changes in kidney weight (B) between mice fed a STD, a HFD or HFD-Losartan. Temporal evolution of blood glucose (C) in mice fed a STD, a HFD or HFD-Losartan. T0
corresponds to Day 0; T1 corresponds to Week 30, just before starting losartan or placebo treatment; T2 corresponds to Week 32; T3 and T4 correspond to Week 34 and 36, respectively. (A) Values are means ± SEM. N=6-8 in each group. Statistical analyses were performed by Two-way ANOVA followed by Newman-Keuls *p ≤ 0.05 HFD and HFD+Losartan versus mice on STD and +p ≤ 0.05 versus T0; (B) Values are means ± SEM. N=6-8 in each group. Statistical analyses were performed by one-way ANOVA followed by Newman-Keuls *p ≤ 0.05 versus mice on STD and #p ≤ 0.05 versus mice on HFD. (C) Values are means ± SEM. N=6-8 in each group. Statistical analyses were performed by Two-way ANOVA followed by Newman-Keuls *p ≤ 0.05 HFD or HFD+Losartan versus mice on STD and +p ≤ 0.05 versus T0.

Figure 2. Determination of the glomerular filtration rate and albuminuria in mice fed a STD, a HFD or HFD-Losartan. Quantitative glomerular filtration rate determined by the renal FITC-inulin clearance expressed in μl/min (A). Values are means ± SEM. N=6-8 in each group. Statistical analyses were performed by one-way ANOVA followed by Newman-Keuls *p ≤ 0.05 versus mice on STD and #p ≤ 0.05 versus mice on HFD.

Quantitative urine albumin/creatinine ratio (UACR) before and after placebo or losartan treatment in mice on STD or HFD at 30 weeks (before) and 36 weeks (after treatment) (B). Values are means ± SEM. N=6-8 in each group. Statistical analyses were performed by one-way ANOVA followed by Newman-Keuls *p ≤ 0.05 versus mice on STD and +p ≤ 0.05 versus mice on STD-placebo.

Figure 3. Sequential filling of cortex and medulla in mice fed a STD, a HFD or a HFD+Losartan.
Representative ultrasound images showing sequential filling of cortex and medulla in kidneys of mice on STD (A), HFD (B), and HFD+Losartan (C). Location of kidney is delineated by dotted white line in t=0 panel; location of cortex delineated in t=0.3 s pane.

**Figure 4.** Time-intensity analysis. Representative normalized time-intensity curves from renal cortex (A) and medulla (B) in mice fed a STD (○), HFD (□), or HFD after Losartan treatment (opened triangle). Raw intensity data without baseline subtraction nor normalization for each mouse cohort is shown for the cortex (C) and medulla (D). Quantitative analysis of the time-to-peak of Cortex (E) and Medulla (F) in STD, HFD or HFD-Losartan. Values are means ± SEM. N=6-8 in each group. Statistical analyses were performed by (A-B) Two-way ANOVA followed by Newman-Keuls *p ≤ 0.05 versus STD and HFD+Losartan and a (E-F) one-way ANOVA followed by Newman-Keuls *p ≤ 0.05 versus mice on STD and #p ≤ 0.05 versus mice on HFD.

**Figure 5.** Determination of peritubular capillaries in mice fed a STD, a HFD or a HFD-Losartan. Semi-quantitative analysis of CD31-positive staining in mice fed a STD, a HFD or a HFD-Losartan at week 36 (A). Representative photomicrographs of CD31 immunostaining (x 200) in mice fed a STD (B), a HFD (C) or a HFD-Losartan (D). Values are means ± SEM. N=6-8 in each group. Statistical analyses were performed by one-way ANOVA followed by Newman-Keuls *p ≤ 0.05 versus mice on STD and #p ≤ 0.05 versus mice on HFD.

**Figure 6.** Determination of inflammatory markers in mice fed a STD, a HFD or HFD-Losartan. Semi-quantitative analysis of CD43-positive cells in renal tissue (A), and quantitative urine hydrogen peroxide level in mice on STD, HFD or HFD-Losartan (B).
Values are means ± SEM. N=6-8 in each group. Statistical analyses were performed by one-way ANOVA followed by Newman-Keuls *p ≤ 0.05 versus mice on STD and #p ≤ 0.05 versus mice on HFD.

**Tables**

**Table 1.** Determination of inflammatory and pro-fibrotic markers in mice fed a STD, a HFD or a HFD-Losartan at week 36

<table>
<thead>
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<th>STD</th>
<th>HFD</th>
<th>HFD-Losartan</th>
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<tr>
<td>Angiotensinogen</td>
<td>1.00 ± 0.14</td>
<td>0.94 ± 0.16</td>
<td>1.10 ± 0.19</td>
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<td>Renin</td>
<td>1.00 ± 0.15</td>
<td>0.82 ± 0.10</td>
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<tr>
<td>Type IV Collagen</td>
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<td><em>1.42 ± 0.14</em></td>
<td>1.04 ± 0.12</td>
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<tr>
<td>MCP-1</td>
<td>1.00 ± 0.18</td>
<td><strong>4.16 ± 1.01</strong></td>
<td>2.73 ± 0.71</td>
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</tbody>
</table>

Quantitative real time PCR was performed with kidney from all groups each normalized against β-actin. Values are means ± SEM. N=6-8 in each group. Statistical analyses were performed by one-way ANOVA followed by Newman-Keuls *p ≤ 0.05 versus mice on STD.
Figure 1

A. 

- **Body Weight** - g
- **T0, T1, T2, T3, T4**
- **STD**, **HFD**, **HFD-Losartan**
- **Losartan treatment**

B. 

- **Kidney Weight/Tibia length** - g/mm
- **STD**, **HFD**, **HFD-Losartan**

C. 

- **Blood Glucose Level** - mg/dl
- **T0, T1, T2, T3, T4**
- **STD**, **HFD**, **HFD-Losartan**
- **Losartan treatment**

* indicates statistical significance at the 0.05 level.

# indicates statistical significance at the 0.01 level.
Figure 2
A

Interlobular arteries
Cortex filling
Medulla filling

B

Delayed cortex filling

C

STD
HFD
HFD-Los
Figure 5.
Figure 6.