Increased Circulatory Lipopolysaccharide From a High Fat Diet Aggravates Inflammation and Exacerbates Renal Failure

Samuel Righi
Virginia Commonwealth University

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Increased Circulatory Lipopolysaccharide From a High Fat Diet Aggravates Inflammation and Exacerbates Renal Failure

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University

By

Samuel Maxfield Righi
Bachelor of Science, Virginia Tech, 2012

Director: Dr. Leon Avery, Ph.D., Physiology
VCU School of Medicine

Virginia Commonwealth University
Richmond, Virginia
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Abstract

INCREASED CIRCULATORY LIPOPOLYSACCHARIDE FROM A HIGH FAT DIET AGGRAVATES INFLAMMATION AND EXACERBATES RENAL FAILURE

By: Samuel Maxfield Righi, MS.

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University

Virginia Commonwealth University, 2014

Program Director: Dr. Leon Avery, Ph.D., Physiology

Kidney failure is frequently associated with the risk factors linked to metabolic syndrome. Lipopolysaccharide (LPS) is a potent inflammatory molecule, which has increased absorption from the gut into blood circulation following a high fat and high-energy diet. We hypothesized that LPS from a high fat diet can amplify inflammation, thereby exacerbating chronic kidney disease and associated disorders. We have found that adding a high fat diet to renal insufficient mice significantly progressed their kidney disease as well as associated disorders, compared to both a high fat diet and renal insufficiency alone. Additionally, we were able to demonstrate in vitro that the combination of LPS and palmitic acid, a marker of high fat diet, induced inflammatory pathways significantly more than either LPS or palmitic acid alone. These results provide insight into connection between a high fat diet and the progression of chronic kidney disease as well as associated disorders.
Introduction

1.1 Chronic Kidney Disease

Kidney failure is frequently associated with the risk factors of metabolic syndrome, including hypertension, glucose intolerance, and cardiovascular disorders such as atherosclerosis. The western style diet, characterized by highly processed foods with high contents of sugar and fat, is a major contributor to diseases associated with metabolic syndrome, such as type 2 diabetes and atherosclerosis, as well as contributing to increased incidences of chronic kidney disease (CKD) (1). In a large cohort of over 320,000 patients it was found that body mass index (BMI) was a strong independent risk factor for end stage renal disease (ESRD), even after adjustment for other major risk factors that are associated with ESRD (2). However, the mechanisms by which metabolic syndrome due to high fat diet exacerbates CKD remain elusive and largely speculative.

In previous studies we have shown that high fat diet can produce atherosclerosis and glucose intolerance in LDL receptor knock out mice (LDLR−/−) (3, 4). Others have shown that partially nephrectomized LDLR−/− mice, a model of renal insufficiency, on a high fat diet experience vascular calcification, osteodystrophy and hyperphosphatemia (5). ApoE knockout mice, another model of atherosclerosis, have accelerated atherogenesis following uremia (6, 7). The major distinction between ApoE knockout and LDLR−/− mice is their response to diet. On a routine chow diet, ApoE knockout mice have high cholesterol (400 to 500 mg/dL) and atherosclerotic lesions, whereas the chow-fed LDLR−/− mice have
mildly increased plasma cholesterol levels (175 to 225 mg/dL) and usually do not develop significant atherosclerotic lesions (8). This suggests that environmental factors, such as diet, govern the development of atherosclerosis and glucose intolerance in LDLR−/− mice. We hypothesized that the nephrectomized LDLR−/− mouse would be a suitable model to elucidate the mechanism by which high fat diet can influence hypertension, renal dysfunction, glucose intolerance and atherosclerosis.

In addition, consumption of high fat diet can cause mild endotoxemia. Studies in both mice and men, show that direct manipulation of the gut microbiome improves features associated with metabolic syndrome and obesity, indicating that microbiota-based interventions are promising new targets for treating some diseases (9-11).

1.2 LPS leakage

Lipopolysaccharide (LPS) is an endotoxin released by gram-negative bacteria that reside in the gut. While LPS usually remains in the gut, there are a variety of factors and conditions that can cause this LPS to seep out and into circulation. This occurs through changes in tight junction proteins, causing LPS to leak through the gut wall (Figure 1). It has been demonstrated that high fat, high-energy diets alter gut microbiota and can cause leaky gut (9, 12). Furthermore, recent studies have also provided considerable evidence that patients and animals with renal failure have altered gut microbiota that can result in increased intestinal permeability, leading to the leakage of inflammatory materials (13-16).
Once LPS is in the circulation it has the ability to bind to the TLR4 complex of a variety of cells that secrete inflammatory activating cytokines. Since both high fat diet and renal insufficiency are causes of leaky gut, we hypothesized that, when these two conditions are combined, a significant increase in LPS leakage from the gut into circulation will be observed. This increase in circulating LPS would further progress chronic kidney disease and associated disorders.

![Figure 1. Diagram of LPS permeability through the gut wall.](image)

Schematic drawing depicting altered tight junction proteins, which allow for LPS permeability through the gut wall into circulation.

### 1.3 Metabolic Syndrome

Metabolic syndrome is characterized by having at least one of the following risk factors: large waistline, high triglyceride levels, low HDL
cholesterol, high blood pressure, and high resting blood glucose. Many of these risk factors accompany patients with chronic kidney disease. Additionally, patients with metabolic syndrome are up to 2.5 times more likely to develop chronic kidney disease than healthy individuals (17). Specifically, hyperglycemia and hypertension are the most influential factors in developing CKD, due to their role in predisposing patients to CKD and CKD progression (18-20). Although there is a strong association between metabolic syndrome and chronic kidney disease, the mechanism for the correlation is unknown. We hypothesize that one of the connections between chronic kidney disease and the risk factors that make up metabolic syndrome is an increase in inflammation. More specifically, we believe LPS-induced inflammation could be an underlying cause for the progression of chronic kidney disease and associated metabolic syndrome disorders.

1.4 Cytokines

A major contributor to the LPS-induced inflammatory signals is the binding of LPS to TLR4 on macrophages. This binding aids in the recruitment of immune cells to the location, as well as inducing the macrophage to secrete inflammatory cytokines that, when in excess, can cause kidney damage (21, 22). When used over short periods of time inflammatory cytokines can be very beneficial in fighting infection and foreign objects in the body; However, having these inflammatory cytokines linger in the body for too long can cause cellular damage and can further exacerbate many inflammatory conditions. Two of the cytokines
that are released via TLR4 activation that we examine in this study are TNF-α and IL-6. We hypothesize that a high fat diet combined with kidney insufficiency increases circulating LPS, thereby increasing TLR4 signaling. Through migration signaling the total number of macrophages in the kidney will be increased, along with the levels of TNF-α and IL-6 secreted by macrophages. Ultimately this will lead to an increase in inflammation, which will further progress CKD and will aggravate associated disorders.

1.5 Inflammation

Inflammation is a biological response by vascular tissue to harmful stimuli including viruses, bacteria, cell damage, and other irritants. Inflammation is caused when immune cells release chemicals in response to these harmful stimuli. The chemicals then initiate a number of complex cascades that cause an increase in blood flow to the specific area, which results in redness and swelling. Short-term inflammation, or acute inflammation, caused by cytokine release can be beneficial for the body to fight an infection. However, when these excess cytokines are present for long periods of time, known as chronic inflammation, they can cause significant harm to the body. Inflammation plays a large role in the body’s response to foreign material, but it also has a major role in a plethora of diseases and the progression of those diseases. Chronic kidney disease is associated with increased levels of inflammatory markers, and elevated inflammation can mediate loss of kidney function in patients with chronic kidney disease (23). Some of the factors of CKD that lead to increased inflammation
include malnutrition caused by decrease in appetite, anemia, and uremia. This increase in inflammation can further progress CKD, which will cause inflammation to rise even more. This ongoing cycle is a major danger associated with CKD and its associated disorders.
Materials and Methods

All antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) unless otherwise stated.

2.1 Animals

The Institutional Animal Care and Use Committees of Virginia Commonwealth University approved all animal procedures. Mice were divided into four groups with six animals in each group. The control group underwent sham surgery, and three other groups underwent 5/8th nephrectomy by the procedure described below.

2.2 Surgery

Renal insufficiency was achieved by 5/8th nephrectomy where a quarter of the left kidney was ligated, followed by the removal of the right kidney 1 week later. The surgical procedure was similar to the 5/6th nephrectomy done previously in our laboratory with slight modifications to achieve 5/8th (24). LDL receptor knock out (LDLR−/−) mice between 8-10 weeks were used in all experiments. All operations were carried out in sterile conditions under isoflurane anesthesia. In brief, a left flank incision was made, exposing the left kidney. The kidney was decapsulated and the lower quarter of the kidney was ligated, leaving the remaining 3/4th of the mass of the left kidney unharmed. The muscle and skin incisions were sutured with polypropylene suture. The animals were returned to the vivarium to recover. One week later, a right flank incision was made, the
renal vessels and ureter were ligated, and the entire right kidney was excised. Animals were returned to the vivarium to recover, and treatment was started after 7 days. The animals were studied for 16 weeks and on the 17th week they were sacrificed.

The animals were divided as follows:

- **Control**: Sham operated on normal chow diet
- **Nx+Chow**: Nephrectomized on normal chow diet
- **HFD**: Sham operated on high fat diet (TD 88137)
- **Nx+HFD**: Nephrectomized on high fat diet (TD 88137)

The normal diet is 5.8% fat by weight, accounting for 17% of the calories. The high fat diet is 21% fat by weight, accounting for 42% of the calories. The main source of fat for the high fat diet is anhydrous milk fat (butterfat). The fatty acid analysis of the anhydrous milk fat is 62.8% saturated fat, 27.3% monounsaturated fat, and 4.7% polyunsaturated fat.

### 2.3 Pair fed high fat diet (PF-HFD)

In previous studies we have observed that nephrectomized animals experience a decrease in food consumption as compared to sham animals after 8 – 10 days. As a result, we corrected for this difference by using a pair fed high fat diet (PF-HFD) group. At the start of treatment, the nephrectomized animals were given a weighed amount of high fat diet around 4 pm and permitted to eat ad lib. The following evening the remaining food was weighed and the average
amount consumed was given to the sham operated animals and this group was designated PF-HFD. Since the food consumption of the sham control was more than the nephrectomized group we added normal chow to the PF-HFD group to prevent malnutrition. On average, the nephrectomized mice consumed 3/4\textsuperscript{th} the amount of food of the control animals.

2.4 Serum Urea Nitrogen, Serum Creatinine and Urinary Albumin

Serum urea nitrogen (urea) and creatinine were measured by a Quantichrom creatinine™ and Quantichrom urea assay™ kit. (Bioassay system; Hayward, CA). Albumin was measured by mouse albumin ELISA assay kit (Bethyl Laboratories; Montgomery, TX).

2.5 Lipopolysaccharide (LPS) measurement

Plasma LPS was analyzed by Lamilus Amebocyte Lysate (LAL) assay (Lonza, Walkersville, Maryland, USA) according to the manufacturer’s instructions, with the following modifications: samples were diluted 5-10-fold to avoid interference with background color and preheated to 70\textdegree C for 10 minutes prior to analyses.

2.6 Longitudinal Measurement of Arterial Pressure by Tail Plethysmography

Arterial pressure (BP) was determined by tail plethysmography as previously described using the CODA 2 system (Kent Scientific, Torrington, CT)
CODA 2 utilizes volume pressure recording sensor technology to measure tail blood pressure. This computerized, noninvasive tail-cuff acquisition system can simultaneously measure systolic, diastolic, and mean arterial pressure without operator intervention. Before surgery, mice were conditioned over a period of 3 days and kept in a restraining holder for a 5- to 10-min period. On the fourth day, BP was recorded (week 0). During this period, 25 sequential readings were obtained. Readings within a range of 10 mmHg were averaged. Two weeks after the second surgery, the animals were retrained and BP was recorded every alternate week. In this study, we report the mean arterial pressure (MAP) of each group.

2.7 Histology

Animals from each group were examined for histological changes in the kidney. A portion of the kidney was cut and fixed in 10% buffered formalin for light microscopy. Sections were cut at 2-µm thickness and stained with periodic acid-Schiff. A basic scoring system was used, as described previously (25). A minimum of 100 glomeruli was scored per animal by an observer blinded to the origin of the tissue.

2.8 Intraperitoneal Glucose Tolerance Tests (IPGTT):

IPGTTs were carried out as described previously (17). Briefly LDLR−/− mice of different cohorts were fasted overnight and given a single bolus of glucose (2 mg/g body weight) intraperitoneally. Blood glucose levels were
determined using a commercially available glucometer and tail vein blood at 0 minutes (before IP injection), 15, 30, 60, and 120 minutes after IP administration.

2.9 Quantitative atherosclerosis analyses.

Quantitative atherosclerotic enface analysis was carried out as described before (26). Briefly the aorta was dissected from the heart to the iliac bifurcation, cleaned of any surrounding tissue, opened longitudinally, pinned on black wax, and fixed for 24 h in 10% buffered formalin. The fixed aortas were imaged on a black background using a Canon digital camera fitted with a 60-mm, f/2.8-macro lens. Total area and area occupied by the lesions in the aortic arch and total aorta were determined using Axiovision Image Analysis software (Carl Zeiss). The person quantifying the area occupied by lesions was blinded to the identity of the images. Extreme care was taken to ensure that any residual adventitial fat that appeared translucent on the images was not included in the area occupied by the lesions that were dense and opaque.

2.10 Homogenization

Each kidney was removed and immediately frozen in liquid nitrogen and kept at −70°C until use. The frozen kidney was ground to a powder and then mixed in ice-cold RIPA buffer (Thermo Fisher Scientific, Rockford, IL) and protease and phosphatase inhibitors. The kidney was homogenized in an ice-chilled Dounce homogenizer at 4°C, and centrifuged at 4°C at 2500 rpm for 5 minutes. The supernatant was aliquoted and stored at −70°C until use.
2.11 Immunoblotting

Kidney homogenates (75–100 µg total protein) were separated on a 4–20% SDS-PAGE gel, and proteins were transferred to a polyvinylidene difluoride membrane as described before (24). Blots were washed briefly in phosphate-buffered saline containing 1% Tween 20 (PBS-T) and blocked in 5% nonfat dry milk, followed by incubation with appropriate antibodies in 5% nonfat dry milk overnight at 4°C. Following three to five washes in PBS-Triton X-100, blots were subsequently incubated with secondary antibody appropriately diluted in 5% nonfat dry milk for 1 hr at room temperature. Blots were washed three to five times in PBS and were developed using Lightning Chemiluminescence Reagent Plus and exposed to X-rays.

2.12 Cell Culture

Transduction with recombinant adenoviruses: NFkB-Luciferase adenovirus (NFkB-adluc) was obtained from Vector Biolabs (Philadelphia, PA). Expression of the luciferase gene is controlled by a synthetic promoter that contains direct repeats of the transcription recognition sequences for the binding sites for NFkB. Mouse macrophages (J774) cultured in DMEM were infected overnight at a multiplicity of infection (MOI) of 5 at 37°C. Cells were exposed to palmitic acid (0, 150 µM, 300 µM and 600 µM), 50 pg/mL LPS (low dose LPS), or 50 pg/mL LPS (high dose LPS), followed by incubation for 4 hours. Cells undergoing a second round of palmitic acid treatment were washed twice with
sterile PBS, followed by palmitic acid treatment (0, 150 µM, 300 µM and 600 µM), and incubation for an additional 4 hours. After respective incubations the cells were lysed and assayed for luciferase activity using the Promega kit.

2.13 Mouse macrophage (J774) for Western Blots

Non-transduced J774 cells cultured in DMEM were treated with palmitic acid (0, 150 µM, 300 µM and 600 µM) and/or LPS as described above. After appropriate incubation time intervals the cells were lysed in RIPA buffer and were centrifuged at 2500 rpm for 5 minutes followed by processing for western blots as described above in the immunoblotting section.

2.14 Isolation of Peritoneal Macrophages:

Thioglycollate-elicited peritoneal macrophages were harvested, and non-adherent cells were removed after 2 hr, and medium was replaced with fresh growth medium (4). For determination of gene expression, total RNA was isolated from adherent macrophages 2 hr after plating using the RNeasy kit from Qiagen.

2.15 Quantitative Real-Time RT-PCR Analysis

Total RNA was extracted from kidneys with the RNeasy Mini Kit as described previously (24). Briefly, 2 µg of total RNA were reverse transcribed with the Thermoscript RT-PCR System (Invitrogen), and first-strand cDNA was used to perform real-time PCR using the Stratagene Mx3000p real-time PCR
system with TaqMan Universal PCR Master Mix and optimized probe and primer sets from Applied Biosystems (Foster City, CA). The following probes were used: CD36 (Mm00432403_m1), SR-A (Mm00446214_m1), IL-6 (Mm00446190_m1) and TNF-α (Mm00443258_m1). The amount of mRNA was calculated by the ΔCT method and normalized to β-actin.

2.16 Statistical Analysis

Statistical comparisons among groups were performed using ANOVA followed by Tukey’s multiple comparison test. Groups were considered to be significantly different at \( p \leq 0.05 \) – \( p \leq 0.001 \).
Results

3.1 Diet and Weight

In previous studies it has been observed that weight loss is associated with renal insufficiency. In order to examine the role of weight in our model, we obtained the average weight of the mice in each treatment group. Mice that were fed a high fat diet experienced a 45% increase in body weight compared to the controls ($p < 0.01$) (Figure 2). The average body weight of control animals was $26.7 \pm 2$ grams, which was 21% higher ($p < 0.01$) than the nephrectomized animal on normal diet (Nx+Chow), but was not significantly different from the nephrectomized animals on high fat diet (Nx+HFD). Two weeks after the surgery we observed that the total intake of food was noticeably less for the nephrectomized animals compared to the control animals. In order to decrease the influence of appetite suppression induced by CKD, we introduced a pair fed control group as described in the methods. The average weight of PF-HFD was

![Figure 2](image_url)  

**Figure 2. Average body weight of mice after the 16-week experiment.** The significant changes are represented by the following signs, *$p < 0.05$ from control; **$p < 0.01$ from control; †$p < 0.05$ from PF-HFD; †† $p < 0.1$ from PF-HFD; # $p < 0.05$ from HFD; ## $p < 0.01$ from HFD; ‡ $p < 0.05$ from Nx+Chow; ‡‡ $p < 0.01$ from Nx+Chow.**
32.9 ± 2.9 gms, which was 17% lower than HFD group but significantly higher than the control, Nx+Chow and Nx-HFD (p < 0.05). The average body weight of Nx+HFD (26.5±5.6 gms) was significantly higher than Nx+Chow (p < 0.05).

### 3.2 Blood Pressure

One of the characteristic risk factors for metabolic syndrome is high blood pressure. To assess the effects of high fat diet and renal insufficiency on blood pressure, we measured the mean arterial pressures (MAP) of the groups over the course of the experiment. For the first six weeks there was not a significant difference between the MAPs of the groups. After six weeks both the Nx+Chow and Nx+HFD MAP started to significantly increase, and remained elevated compared to the other groups. Figure 3 displays the longitudinal changes in MAP for the groups over the 16-week period. The MAP of the control cohort did not show a significant change over the course of the experiment. It ranged from 95 ± 6.3 mm Hg at the start of the experiments to 102 ± 5.4 mm Hg, at 16 weeks. The high fat diet given ad lib (HFD; data not shown) and pair fed high fat diet (PF-HFD) were not significantly different from each other or the control at the end of the 16-weeks. The mean MAP of Nx+Chow at 6, 12 and 16 weeks were 107 ± 2.7 mm Hg, 134 ± 19.6 mm Hg, and 144 ± 18.6 mm Hg respectively. The mean MAP of Nx+HFD at 6, 12 and 16 weeks were 111.5 ± 2.380, 154.0 ± 5.89 mm Hg, and 164.5 ± 6.19 mm Hg. Between 6 to 16 weeks the MAP of Nx+Chow and Nx+HFD were significantly higher than control and PF-HFD (p < 0.01). After 8 weeks the MAP of Nx+HFD was consistently higher than the Nx+Chow, however
the difference was not statistically significant.

3.3 Urinary Albumin Creatinine Ratio (ACR)

The ratio of albumin to creatinine is an indicator of many conditions known to be associated with metabolic syndrome, and additionally can be affected by kidney dysfunction. As such, we measured the urinary albumin creatinine ratio (ACR) through the duration of our experiment. Over the course of the first four-weeks there was not a significant difference in ACR between the groups. From 4-weeks on, all of the treatment groups had an ACR significantly higher than the control group. At 8 weeks the HFD, NX-Chow, and NX-HFD groups had ACR levels significantly more than the PF-HFD group. From 12-weeks to 16-weeks

![Figure 3. Mean arterial pressures (MAP) plotted over the 16-week experiment. The significant changes are represented by the following signs, *p < 0.05 from control; **p < 0.01 from control; †p < 0.05 from PF-HFD; †† p < 0.1 from PF-HFD; # p < 0.05 from HFD; ## p < 0.01 from HFD; ‡ p < 0.05 from Nx+Chow;; and ‡ ‡ p < 0.01 from Nx+Chow.](image-url)
the ACR levels were from lowest to highest as follows: control, PF-HFD, HFD, NX-Chow, and NX-HFD. All of the ACR values of the groups were significantly different from one another at the end of the 16 weeks as displayed in Figure 4. At the end of 16 weeks the ACR of control, HFD, PF-HFD, Nx+Chow and Nx+HFD were 6.2 ± 1.52, 91.5 ± 9.91, 36.7 ± 7.09, 233.7 ± 63.70, and 391.2 ± 59.64 respectively. The significant differences among the groups at the end of 16 weeks are as follows NX+HFD > Nx+Chow (p < 0.05); Nx+Chow > HFD (p < 0.05) and HFD > PF-HFD (p < 0.01). All the groups were significantly higher than control.

![Figure 4. Urinary albumin creatinine ratio (ACR) plotted over the 16-week experiment. The significant changes are represented by the following signs, *p < 0.05 from control; **p < 0.01 from control; †p < 0.05 from PF-HFD; †† p < 0.1 from PF-HFD; # p < 0.05 from HFD; ## p < 0.01 from HFD; ‡ p < 0.05 from Nx+Chow;; and ‡ ‡ p < 0.01 from Nx+Chow.](image)

3.4 Serum Urea and Serum Creatinine

In order to assess the level of kidney function in our mice, we measured serum urea and serum creatinine levels at the end of the treatment, as
The serum urea and creatinine of the animals on PF-HFD (data not shown) were not significantly different from HFD and control. The serum urea of the Nx+Chow group (36.4 ± 5.6 mg/dL) was more than 45% higher than control and PF-HFD (p < 0.05). The serum urea of Nx+HFD (55.5 ± 16.1 mg/dL) was about 60% higher than the control and PF-HFD (p < 0.01) and significantly higher than Nx+Chow (p < 0.05). As shown in Figure 6, serum creatinine of the control (0.45 ± 0.07 mg/dL) was not significantly different from PF-HFD (0.5 ± 0.09 mg/dL). Serum creatinine levels of Nx+Chow (0.7 ± 0.04 mg/dL) were significantly higher than both control and PF-HFD groups (p < 0.05). The creatinine level of Nx+HFD group (0.9 ± 0.04 mg/dL) was also significantly higher than the control and the PF-HFD cohorts (p < 0.01). In addition, the serum creatinine of Nx+HFD was significantly higher than Nx+Chow (p < 0.05). These data suggest that a high fat diet may not induce renal failure by itself, but has the capability of intensifying renal insufficiency.

Figure 5. Serum urea levels after 16-weeks. The significant changes are represented by the following signs, *p < 0.05 from control; **p < 0.01 from control; †p < 0.05 from PF-HFD; †† p < 0.1 from PF-HFD; # p < 0.05 from HFD; ## p < 0.01 from HFD; ‡ p < 0.05 from Nx+Chow; and ‡ ‡ p < 0.01 from Nx+Chow.
3.5 Histology

Previous studies in the lab with 5/6th nephrectomized rats displayed global and segmental sclerosis with tubular dilation and atrophy (24, 25). In contrast, our 5/6th nephrectomized mice displayed only large vacuoles and protein droplets but no significant sclerosis or tubular damage (data not shown) were present. This suggests that this is a model of renal insufficiency, rather than renal failure.

3.6 Lipopolysaccharide (LPS) and Macrophages

LPS is an endotoxin released by gram-negative bacteria, which elicits an immune response in animals, including inflammation. Both humans and mice have increased absorption of LPS from the gut into the circulatory system following a high fat/high energy diet (27). To this end, we measured LPS serum levels for all groups at the end of the 16-week experiment, as displayed in

![Figure 6. Serum creatinine levels after 16-weeks. The significant changes are represented by the following signs, *p < 0.05 from control; **p < 0.01 from control; †p < 0.05 from PF-HFD; †† p < 0.1 from PF-HFD; # p < 0.05 from HFD; ## p < 0.01 from HFD; ‡ p < 0.05 from Nx+Chow; and ‡‡ p < 0.01 from Nx+Chow.](image)
Figure 7. The serum LPS levels for the control group (0.4 ± 0.07 EU/mL) were significantly less than the other treatment groups. The PF-HFD group serum LPS levels (1.57 ± 0.29 EU/mL) were significantly more than the controls, however they were significantly less than the other three groups. The HFD (3.00 ± 0.40 EU/mL) and NX-Chow (2.34 ± 0.35 EU/mL) treatment groups had serum LPS levels significantly more than the control and PF-HFD groups, but they were not significantly different from one another. The NX-HFD treatment group had the highest serum LPS levels (4.13 ± 0.74 EU/mL), and they were significantly above the other groups. The relationship of the serum endotoxin levels between the groups are as follows: Nx+HFD > HFD > Nx+Chow = PF-HFD. These data suggest that renal insufficiency can affect serum LPS levels. Furthermore, the endotoxin levels appear to increase due to the consumption of high fat diet.

Figure 7. Serum LPS levels in mice at the end of 16 weeks. At the end of 16 weeks the animals were sacrificed and serum lipopolysaccharide (LPS) was measured by Lonza Assay kit. The significant changes are represented by the following signs, *p < 0.05 from control; **p < 0.01 from control; †p < 0.05 from PF-HFD; †† p < 0.1 from PF-HFD; # p < 0.05 from HFD; ## p < 0.01 from HFD; ‡ p < 0.05 from Nx+Chow; † and †† p < 0.01 from Nx+Chow.
Bacterial endotoxin LPS, can activate migration of macrophages to various organs by binding to the macrophage TLR4 receptors (28). The influx of macrophages in the kidney was evaluated by quantitation of macrophage markers CD-68 and SR-1 in the kidney by RT-PCR. As seen in Figures 8 and 9, all the groups (HFD, PF-HFD, Nx+Chow and Nx+HFD) had higher macrophage markers than the control. The maximum influx of macrophages in the kidney was in the Nx+HFD group followed by Nx+Chow. Both the macrophage markers in the Nx+HFD group were 1.5 times higher than the Nx+Chow (p < 0.05). The macrophage influx in the Nx+HFD and Nx+Chow was not only higher than PF-HFD (p < 0.01) but was also significantly higher than HFD (p < 0.05) suggesting renal injury promotes higher macrophage infiltration in LDLR−/− mice. This influx of macrophages following renal injury can be aggravated by high fat diet.

Figure 8. Macrophage marker CD 68 was measured using QPCR. The level of this maker represents the magnitude of macrophage influx in the kidney. The significant changes are represented by the following signs, *p < 0.05 from control; **p < 0.01 from control; †p < 0.05 from PF-HFD; †† p < 0.1 from PF-HFD; # p < 0.05 from HFD; ## p < 0.01 from HFD; † † p < 0.05 from Nx+Chow; ‡ p < 0.01 from Nx+Chow.
There is a strong correlation between inflammation and renal failure (29). Specific cytokines released by macrophages during an immune response can lead to increased inflammation. To examine the role of cytokines during renal failure we looked at the levels of inflammatory cytokines IL-6 and TNFα in the kidney. The increase in kidney TNFα of Nx+HFD, Nx+Chow HFD and PF-HFD from the control were 10 fold, 5.8 fold, 3.7 fold, and 2.6 fold respectively. Figure 10 shows that all of the treatments groups had significantly higher levels of TNFα compared to the control. The highest levels of TNFα came from the Nx+HFD group, which was significantly higher than rest of the groups. The TNFα levels in the kidney of PF-HFD were significantly lower than HFD (p < 0.05) and Nx+Chow (p < 0.01). The TNFα in the Nx+Chow kidney was significantly higher than HFD (p < 0.05).
The increase in kidney IL-6 of Nx+HFD, Nx+Chow HFD and PF-HFD from the control was 7.3 fold, 4.2 fold, 2.7 fold, and 2.4 fold respectively. As shown in Figure 11, they were all significantly higher than the control. Similar to TNFα, the IL6 levels were the highest in the Nx+HFD group, and they were significantly higher than rest of the groups as displayed by Figure 11. Also, the IL-6 levels in Nx+Chow were significantly higher than HFD (p < 0.05) and PF-HFD (p < 0.05).

![Graph showing kidney TNFα levels measured by QPCR at the end of 16 weeks.](image)

**Figure 10. Kidney TNFα levels measured by QPCR at the end of 16 weeks.** The significant changes are represented by the following signs, *p < 0.05 from control; **p < 0.01 from control; †p < 0.05 from PF-HFD; †† p < 0.1 from PF-HFD; # p < 0.05 from HFD; ## p < 0.01 from HFD; and ‡ p < 0.05 from Nx+Chow.
We also looked at the cytokine levels of peritoneal macrophages activated through treatment with thioglycolate. As seen in Figures 12 and 13, the changes in both the cytokines in the macrophages were qualitatively similar to the kidney cytokines. It is possible that the increased influx of macrophages contributed to the increase in cytokine levels; however, during this study we did not determine the precise influence that macrophages alone had on the increased kidney cytokine levels.

Figure 11. Kidney IL-6 levels measured by QPCR at the end of 16 weeks. The significant changes are represented by the following signs, *p < 0.05 from control; **p < 0.01 from control; †p < 0.05 from PF-HFD; †† p < 0.1 from PF-HFD; # p < 0.05 from HFD; ## p < 0.01 from HFD; and ‡ p < 0.05 from Nx+Chow.
Figure 12. Activated macrophage TNFα levels measured by QPCR. Thioglycolate elicited macrophages were plated as described in the methods, and TNFα levels were measured by QPCR. The significant changes are represented by the following signs, *p < 0.05 from control; **p < 0.01 from control; †p < 0.05 from PF-HFD; †† p < 0.1 from PF-HFD; # p < 0.05 from HFD; ## p < 0.01 from HFD; and ‡ p < 0.05 from Nx+Chow.

Figure 13. Activated macrophage IL-6 levels measured by QPCR. Thioglycolate elicited macrophages were plated as described in the methods, and IL6 levels were measured by QPCR. The significant changes are represented by the following signs, *p < 0.05 from control; **p < 0.01 from control; †p < 0.05 from PF-HFD; †† p < 0.1 from PF-HFD; # p < 0.05 from HFD; ## p < 0.01 from HFD; and ‡ p < 0.05 from Nx+Chow.
3.8 Intraperitoneal Glucose Tolerance Tests (IPGTT)

High fat diet and renal failure are usually accompanied by hyperglycemia. To this end, we carried out intraperitoneal glucose tolerance tests (IPGTTs). Figure 14 depicts the blood glucose levels of the groups at various time intervals. In our IPGTT study the blood sugar of the control at 0 hour (before IP glucose administration) was significantly lower than the rest of the cohorts (p < 0.05-0.01). The blood glucose of the Nx+HFD cohort was significantly higher than the rest of the groups at all-time points. 15 minutes after glucose administration, the NX-HFD group was the only treatment group that was significantly higher than the control. After 30 minutes the NX+Chow group had glucose levels significantly higher than the control. The HFD and NX-HFD had significantly higher glucose levels than the NX+Chow, PF-HFD, and control. After 60 minutes the NX+Chow and PF-HFD groups had glucose levels remained significantly higher than the control, and both the HDF and NX-HFD groups were significantly more than the NX+Chow and PF-HFD. After 90 minutes the NX+Chow, PF-HFD, and HFD groups had significantly increased glucose levels compared to the control, but were not significantly different from one another. The NX-HFD group had glucose levels significantly more than all the other groups.
Figure 14. Blood glucose concentration after intraperitoneal injection of 2mg/g glucose. After overnight fasting the tail was clipped and blood glucose was measured by a commercial glucometer for the 0 hour sample. Following the 0 hour sample 2 mg/g glucose was administered and blood concentration versus time is shown. The significant changes are represented by the following signs, *p < 0.05 from control; **p < 0.01 from control; †p < 0.05 from PF-HFD; †† p < 0.1 from PF-HFD; # p < 0.05 from HFD; ## p < 0.01 from HFD; and ‡ p < 0.05 from Nx+Chow.

Figure 15. Area under the curve of blood glucose levels from 0-120 minutes. The significant changes are represented by the following signs, *p < 0.05 from control; **p < 0.01 from control; †p < 0.05 from PF-HFD; †† p < 0.1 from PF-HFD; # p < 0.05 from HFD; ## p < 0.01 from HFD; and ‡ p < 0.05 from Nx+Chow.
Figure 15 shows the area under the curve of glucose concentration versus time (0-120 minutes) calculated for each cohort. The Nx+HFD group was significantly higher than its pair fed control (PF-HFD; p < 0.01) and was even higher than HFD group (p < 0.05) and Nx+Chow (p < 0.05). Although the AUC of Nx+Chow was higher than control it was not significantly different from PF-HFD.

3.9 Atherosclerotic Lesions

Increased rates of cardiac death are a common feature in patients with renal failure. End stage renal failure patients have been shown to have enlarged coronary plaques compared to non-uremic patients, and this could contribute to the increased cardiac complications in renal failure patients (30). To test the impact of our treatments on cardiovascular health we examined the percent area of the aorta covered with atherosclerotic lesions by using en face analyses. Figure 16 shows representative images of aortae obtained from HFD, PF-HFD and Nx+HFD groups. The control animals and Nx+Chow group did not show any lesions. Therefore these groups were not included in our analyses. In the HFD group 20.2 ± 3.7 % area of the aortae were covered by lesions as seen in Figure 17. Although the Nx+HFD (25.5 ± 6.9 % lesion area) had more lesions than HFD, it was not significantly different. In our preliminary study we had observed this effect and realized that the food consumption of Nx+HFD was significantly lower than the non-nephrectomized HFD group. Since diet influences the extent of atherosclerosis, we introduced a pair fed high fat diet group (PF-HFD) as described in the methods. This PF-HFD group allowed us to evaluate the effect
that renal failure alone had on atherosclerosis by feeding two separate groups the same amount of high fat diet by weight. This allowed us to deduce that any changes that were seen were due to renal failure and not from the high fat diet. There was no significant difference in the lesion area between HFD and PF-HFD. However, the aortic lesions of the Nx+HFD were 1.8 fold higher than PF-HFD (p < 0.01) suggesting that the diet influenced the aortic lesions in the animals with renal insufficiency.

Figure 16. En face pictures of the atherosclerotic lesion in the arteries and analysis of the lesion area.
3.10 *In vitro* study to determine interaction of Palmitic acid (PA) and LPS

The level of activation of NFkB was assessed in order to measure inflammatory response. Mouse macrophages (J774) were transduced with adenoviral NFkB with a luciferase reporter and treated with different concentrations of PA and LPS. Figure 18 and Figure 19 show the changes in NFkB luciferase activity with various concentrations of PA and LPS, and LPS alone. Figure 20 shows the changes in NFkB luciferase activity following PA and LPS treatment. The lower dose of LPS (50 pg/mL) did not significantly affect NFkB luciferase reporter activity. The higher dose of LPS (50 ng/mL) caused a drastic increase in NFkB luciferase reporter activity, 33 fold higher ($p < 0.001$) than the control. In humans, after consumption of 1 gram of fat per kg of body
weight, plasma levels of PA (between 2-8 hours) ranged from 100 µM to 600 µM (31). As a result, we measured the ability of various physiologically relevant concentrations of PA, combined with a high fat diet, to activate NFkB. J774 cells were treated with 150 µM PA, 300 µM PA and 600 µM PA in the presence or absence of low dose LPS (50 pg/mL). As seen in Figure 19, compared to control the activation of NFkB by low dose LPS alone was not significantly different. Activation of NFkB by 150 µM PA alone was not significantly different from the control, however 300 µM and 600 µM had 5 fold \((p < 0.05)\) and 11 fold \((p < 0.01)\) higher NFkB luciferase activity respectively. Furthermore, when the cells were exposed to low dose LPS and subsequently treated with 150 µM PA, the increase in NFkB luciferase was 5 fold higher than cells treated with the same concentration of PA without LPS exposure \((p < 0.01)\). Similarly the cells treated with LPS and given 300 µM PA had 3 fold higher NFkB luciferase than cells treated with 300 µM PA alone \((p < 0.05)\), clearly showing that even small doses of LPS exaggerate fatty acid mediated inflammation.
Figure 18. NFkB adeno viral activation by PA. The significant changes are represented by the following signs: ** P < 0.01 Vs control; ‡ P < 0.01 vs 150 µM PA; #p < 0.01 vs 300 µM PA; and †† p < 0.01 vs LPS 50 ng.

Figure 19. NFkB adeno viral activation by LPS. The significant changes are represented by the following signs: ** P < 0.01 Vs control; ‡ P < 0.01 vs 150 µM PA; #p < 0.01 vs 300 µM PA; and †† p < 0.01 vs LPS 50 ng.
We further confirmed this by looking at the phosphorylation of NFkB by immunoblot analysis. Increased phosphorylation i.e. increased ratio of pNFkB/NFkB, reflects augmented inflammation. Figure 21 and Figure 22 show the changes in pNFkB/NFkB ratio with various concentrations of PA and LPS alone. Figure 23, shows the changes in the pNFkB/NFkB ratio when the PA treated cells were pretreated with a low dose of LPS. Figure 22 shows PA was able to independently increase phosphorylation of NFkB. The higher concentrations of PA (300 µM and 600 µM) were significantly higher than the control ($p < 0.05$ and $p < 0.01$ respectively). Compared to the cells treated only with PA, cells exposed to low dose LPS followed by 150 and 300 µM PA had 2-fold higher NFkB phosphorylation ($p < 0.01$). The results of the immunoblot experiments were qualitatively similar to the adenoviral study.

**Figure 20. NFkB adenoviral activation by PA and/or LPS.** The significant changes are represented by the following signs: ** $P < 0.01$ Vs control; †† $P < 0.01$ vs 150 µM PA; # $p < 0.01$ vs 300 µM PA; and ††† $p < 0.01$ vs LPS 50 ng.
Figure 21. NFkB phosphorylation induced by PA. The significant changes are represented by the following signs: ** $P < 0.01$ Vs control; ‡ $P < 0.01$ vs 150 µM PA; # $p < 0.01$ vs 300 µM PA; and †† $p < 0.01$ vs LPS 50 ng.

Figure 22. NFkB phosphorylation induced by LPS. The significant changes are represented by the following signs: ** $P < 0.01$ Vs control; ‡ $P < 0.01$ vs 150 µM PA; # $p < 0.01$ vs 300 µM PA; and †† $p < 0.01$ vs LPS 50 ng.
Figure 23. NFkB phosphorylation induced by PA and/or LPS. The significant changes are represented by the following signs: ** P < 0.01 Vs control; ‡ P < 0.01 vs 150 µM PA; #p < 0.01 vs 300 µM PA; and †† p < 0.01 vs LPS 50 ng.
Discussion

4.1 Impact on Overall Kidney Function

In order to determine the magnitude of impact that a high fat diet combined with renal insufficiency has on overall kidney function we measured serum urea and serum creatinine levels. The serum urea and creatinine levels obtained after the 16-week experiment are displayed in Figures 5 and 6. The high fat diet alone was unable to significantly alter kidney function as indicated by the resulting serum urea and serum creatinine levels. As expected, when nephrectomy was performed the overall kidney function was significantly decreased. The serum urea and creatinine levels in the NX-Chow group were significantly higher than the levels seen in both the control and HFD groups. The most substantial impairment of kidney function occurred when a high fat diet was combined with nephrectomy. The NX-HFD group’s serum creatinine and urea levels were significantly higher than the three other treatment groups. These results indicate that a high fat diet alone cannot diminish kidney function, but when combined with an already compromised kidney it can intensify the kidney dysfunction. This also suggests that healthy kidneys performing optimally are able to handle the stresses accompanied by a high fat diet, but when there is a decrease in kidney function the stresses from the high fat diet are overwhelming and aggravate the decrease in kidney function.

4.2 Metabolic Syndrome

Kidney failure is often coupled with hypertension, glucose intolerance,
cardiovascular disorders, as well as other conditions that make up metabolic syndrome. We wanted to elucidate how a high fat diet during renal insufficiency could affect some of the conditions that make up metabolic syndrome. To do this we collected data on body weight, blood pressure, albumin creatinine ratio, glucose tolerance, and atherosclerotic lesions from the mice.

Excess weight, especially in the form of abdominal visceral fat is one of the main risk factors for metabolic syndrome as well as many other health conditions. Decrease in appetite due to renal insufficiency can lead to weight loss, and this weight loss has been seen previously in our lab in rats suffering from CKD (24). In this study we observed that the average body weight of control animals was significantly higher than the nephrectomized animal on a normal diet, but was not significantly different from the nephrectomized animals on high fat diet. Excess weight can cause increases in inflammation, blood pressure, and atherosclerotic lesions, affect glucose tolerance, and aggravate many other conditions. The uremic animals on normal diet had significant weight loss, which was offset by high fat diet; however, the weight gain of Nx+HFD animals was still significantly lower than the pair fed group. This suggests that diet contributed more than weight gain in inducing the metabolic changes. Renal insufficiency can cause a variety of metabolic changes, but these changes are due to outcomes other than weight gain.

Similar to human patients with CKD, our renal insufficient mouse model displayed significantly increased blood pressure after the 16-week experiment. Having an increase in blood pressure can cause too much stress on the blood
vessels in the kidneys, which can cause damage and decrease function. Nephrectomy alone was able to increase the MAP significantly more than both the control and the PF-HFD groups. The NX-HFD group had the highest MAP out of all the cohorts at the end of the experiments, and they were significantly higher than the rest of the treatment groups. These results demonstrate that a high fat diet alone did not have a significant impact on MAP, but nephrectomy alone did. When a high fat diet was added to renal insufficient mice it was able to increase blood pressure significantly compared to the NX-Chow group. This suggests that healthy kidneys are not overwhelmed by a high fat diet in terms of its affect on MAP, but when that same high fat diet is given to renal insufficient mice there is a significant increase in blood pressure. Damaged kidneys are not able to deal with the added stresses that come from a high fat diet, and this can result in an increase in blood pressure. Our mouse model showed that renal insufficiency can cause an increase in blood pressure, and this increase in blood pressure can further exacerbate the renal insufficiency. This cycle can cause drastic and dangerous decreases in overall kidney health and kidney function.

The albumin creatinine ratio (ACR) is an indicator of many conditions associated with metabolic syndrome including cardiovascular disease, hypertension, glucose intolerance, and many others. Kidney dysfunction can cause albumin to leak into the urine and this will result in an increased ACR ratio. Although kidney failure is one way to increase ACR, many of the other indicators of metabolic syndrome, diabetes for example, have the ability to increase ACR. Our results indicate that a high fat diet alone is able to significantly increase the
ACR compared to the control. These results were expected due to the fact that a high fat diet alone can increase blood pressure, glucose intolerance, and cardiovascular disease. The NX-chow group had a significant increase in ACR compared to the control, HFD, and PF-HFD group. This demonstrates that kidney insufficiency alone has a bigger impact on the ACR than diet alone. When a high fat diet was added to renal insufficiency this created the highest ACR levels and they were significantly higher than all the other groups. This once again indicates that damaged kidneys are not able to cope with the stresses of a high fat diet, and this can causes dramatic metabolic and physiological changes.

Figures 5 and 6 display that a high fat diet alone was unable to decrease kidney function (no significant change in either serum urea or creatinine compared to the control), however it was able to significantly increase the ACR. This denotes that the increase in ACR caused by the high fat diet is the result of changes other than kidney dysfunction.

High fat diet is known to cause glucose intolerance and atherosclerosis in LDLR\(^{-/-}\) mice. We wanted to examine how this high fat diet combined with renal insufficiency would affect glucose intolerance and atherosclerosis. Figure 14 displays the blood glucose levels of the groups at various time intervals, and Figure 15 displays the area under the curve (AUC) for each treatment group. After 120 minutes the HFD, PF-HFD, and NX-chow groups had glucose levels that were significantly increased compared to the control, but they were not significantly different from one another. This demonstrates that a high fat diet alone, and renal insufficiency alone, have the capacity to significantly decrease
glucose tolerance. When the high fat diet was combined with renal insufficiency it resulted in the highest glucose levels of all the treatment groups. Some of the roles the kidney has in glucose regulation involve releasing glucose into circulation by gluconeogenesis, uptake of glucose from the circulation for energy, and reabsorption of glucose in the proximal tubule. The NX-HFD group has significantly higher glucose levels compared to all the other groups starting as early as 15 minutes after glucose administration, until the end of the 2-hour experiment. Our results form the serum creatinine and serum urea levels indicated that a high fat diet combined with renal insufficiency resulted in a significant decrease in overall kidney function. This decrease in overall kidney function could explain the kidneys decrease in ability to regulate blood glucose levels. From these data it can be speculated that high fat diet is not only a strong inducer of glucose intolerance but can play a major role in aggravating hyperglycemia in renal failure.

Using en face analysis we were able to determine the percentage of the aorta that was obstructed by atherosclerotic lesions. There was no statistical difference between the HFD and the PF-HFD groups. The NX-HFD group was also not significantly different from the HFD group, but the percentage occluded by lesions was significantly higher in the NX-HFD group than the PF-HFD group. Both of these groups were fed the same amount of the high fat diet, the only difference between them was the NX-HFD group was renal insufficient. These data emphasizes the importance of the kidneys for lipid metabolism and regulation of circulating lipids. The kidneys have a high capacity for uptake of
lipid binding proteins and lipid regulating hormones (32). The decrease in kidney function observed in these mice could impair the kidneys ability to uptake lipids from the circulation, which would increase the total circulating blood lipids, thus increasing the chances of atherosclerotic lesions.

4.3 Inflammation

LPS is an endotoxin released by gram-negative bacteria, which elicits an immune response in animals, including inflammation. During an immune response, macrophages will migrate to the specific area needed and help mediate multiple processes including inflammation through cytokine release. We examined how our treatments influenced serum LPS levels, influx of macrophages to the kidney, the levels of cytokines in the kidney, and the amount of cytokines produced by activated macrophages.

Blood samples were taken from each of the mice after the experiment was completed to test for serum LPS levels. Figure 7 demonstrates that all of the treatment groups had elevated serum LPS levels compared to the control. The HFD group had serum LPS levels significantly higher than the control and the PF-HFD group. This was expected based on previous research showing both humans and mice have increased absorption of LPS from the gut into the circulatory system following a high fat/high energy diet (27). The only difference between the HFD and the PF-HFD group was the amount of the high fat diet they consumed, so these data emphasize the impact that a high fat diet can have on serum LPS levels. The NX group had serum LPS levels that were significantly
raised above the control group and the PF-HFD group, but they were not significantly different than the HFD group. Our data suggest that both renal insufficiency as well as a high fat diet can significantly increase LPS levels. When a high fat diet is combined with renal insufficiency the highest serum LPS levels were observed. They were significantly higher than all other treatment groups. Both renal damage as well as a high fat diet can increase the absorption of LPS from the gut into circulation, so it was expected that when these two treatments were combined it would produce the highest serum LPS levels.

In order to quantify the influx of macrophages into the kidney we evaluated the levels of macrophage markers CD-68 and SR-1 using RT-PCR. Figures 8 and 9 show that both of the markers had almost identical results for each of the treatments. All of the treatments had increased macrophage markers compared to the control. By binding to the TLR4 receptor of macrophages, LPS can activate the migration of macrophages to various organs in the body (28). The HFD group and the PF-HFD group had significantly different serum LPS levels, but their macrophage markers were not significantly different. This could suggest that LPS does play a role in macrophage migration. But other pathways could have a more substantial impact on the migration. The macrophage marker results followed a very similar path as the serum urea and creatinine levels. For both experiments the two treatments that had the most impact on macrophage influx and kidney function were the NX-Chow and NX-HFD group. This suggests that overall kidney function plays a major role in recruitment of macrophages. Similar to wound healing, macrophage influx to the kidneys is vital to the sterilization and
debridement of the tissue, followed by repair and rebuilding of the tissue (33, 34).

The high fat diet alone causes some damage to kidney tissues and function, but nephrectomy has a more critical affect on tissue damage and kidney function. High fat diet combined with renal insufficiency resulted in the biggest decrease in kidney function, as well as the largest influx of macrophages to the kidney. The high fat diet has the capability to further exacerbate kidney dysfunction during renal insufficiency, and this can increase tissue damage, which results in the influx of macrophages to the kidneys to help with repair.

Cytokine release by macrophages activates the inflammatory pathway during an immune response. We assessed how our treatment groups affected the levels of TNF-α and IL-6 in the kidney, and also in activated macrophages. The results for both the TNF-α and the IL-6 were almost identical in the kidney and macrophages. For the kidney and the macrophages all the treatment groups were significantly higher than the control, and the NX-HFD groups had the highest cytokine levels that were significantly higher than the rest. The NX-Chow groups had significantly raised cytokine levels compared to the HFD. A high fat diet can cause an increase in cytokines in the kidneys and macrophages, but renal insufficiency plays a more crucial role in cytokine release. When a high fat diet is added to renal insufficiency it is able to exacerbate and further increase cytokine release in macrophages, and increase cytokine levels in the kidney. These four experiments compliment each other on the affects that occur when a high fat diet is present during renal insufficiency. Serum LPS levels are increased, there is an influx of macrophages to the kidneys, and these
macrophages produce more cytokines resulting in an increase in kidney cytokine levels, which ultimately leads to a larger inflammatory response.

Both PA and LPS activate inflammatory pathways. Therein they play a key role in disorders such as insulin resistance, CKD and atherosclerosis. Consumption of high fat diet not only leads to the increased blood levels of fatty acids such as PA but also increases absorption of LPS from the gut (9, 31). Although the amount of LPS generated from high fat diet does not cause pronounced inflammation such as seen in septic shock, low levels or sub-clinical levels of LPS, which can be generated following chronic consumption of high fat diet, can sensitize cells to produce significant inflammation (35, 36). Mouse macrophages (J774) were transduced with adenoviral NFkB with a luciferase reporter and treated with different concentrations of PA and LPS. Assessing the activation of NFkB was performed to measure inflammatory response. We used three different concentrations of PA, and two different concentrations of LPS. The three concentrations of PA that we used are similar to those found in humans on a high fat diet. The low concentration of LPS we used is also similar to levels seen in humans on a high fat diet, and the high concentration of LPS was used to demonstrate the inflammatory activating capability of LPS through NFkB activation. Figure 20 shows that low levels of LPS do not elicit much of an inflammatory response alone, but can markedly exaggerate the inflammatory potential of various concentrations of PA. The 150 μM of PA and the low dose of LPS by themselves were not able to significantly increase NFkB activation compared to the control. When the 150 μM of PA was first primed by 50 pg/mL of
LPS the NFkB activation increased significantly more than the control and the 150 µM PA by itself. Similar results occurred when the 300 µM of PA was first primed with 50 pg/mL of LPS. The 300 µM PA by itself was able to activate NFkB significantly more than the control, but when primed with 50 pg/mL of LPS the NFkB activation increased significantly more than 300 µM PA by itself.

To confirm these results we looked at the phosphorylation of NFkB by immunoblot analysis. Increased phosphorylation, i.e. increased ratio of pNFkB/NFkB, reflects augmented inflammation. The results we obtained were very similar to the adenoviral study we performed with NFkB luciferase activation. The pNFkB/NFkB Ratio was not statistically different between the 150 µM PA by itself and the control. When the 150 µM PA was primed with 50 pg/mL of LPS the ratio significantly increased more than the control and the 150 µM PA alone. Similar results occurred when the 300 µM of PA was first primed with 50 pg/mL of LPS. The 300 µM PA by itself was able to increase the pNFkB/NFkB ratio significantly more than the control, but when primed with 50 pg/mL of LPS the pNFkB/NFkB was further increased to be significantly more than 300 µM PA by itself. This demonstrates that when PA is primed with low doses of LPS it results in an increased pNFkB/NFkB ratio, which ultimately leads to a greater inflammatory response.

These results suggest that PA from high fat diet can activate NFkB and inflammation, which can be significantly aggravated by LPS released in the circulation after high fat diet. These low levels of LPS are not enough to initiate an inflammatory response through NFkB activation, but it seems as though they
might sensitize the TLR4 to other effectors. LPS has shown to increase TLR4 expression in adipocytes, and a similar mechanism could also be occurring in macrophages (37).

4.4 Conclusion

The results from our studies showed a high fat diet combined with renal insufficiency significantly aggravated kidney disease and associated disorders compared to both a high fat diet and renal insufficiency alone. Additionally, we were able to demonstrate in vitro that the combination of LPS and palmitic acid, a marker of high fat diet, induced inflammatory pathways significantly more than either LPS or palmitic acid alone. These results support the hypothesis that LPS from a high fat diet is able to amplify inflammation, thereby exacerbating chronic kidney disease and its associated disorders. These results provide insight into connection between a high fat diet and the progression of chronic kidney disease as well as associated disorders.
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