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Research Paper

High circulatory leptin mediated NOX-2-peroxynitrite-miR21 axis activate mesangial cells and promotes renal inflammatory pathology in nonalcoholic fatty liver disease

Firas Alhasson[a,1], Ratanesh Kumar Seth[a,1], Sutapa Sarkar[a], Diana A. Kimono[a], Muayad S. Albadrani[a], Diptadip Dattaroy[a], Varun Chandrashekaran[a], Geoffrey I. Scott[a], Samir Raychoudhury[b], Mitzi Nagarkatti[c], Prakash Nagarkatti[c], Anna Mae Diehl[d], Saurabh Chatterjee[a,*]

[a] Environmental Health and Disease Laboratory, Department of Environmental Health Sciences, Arnold School of Public Health, University of South Carolina, Columbia, SC, USA
[b] Department of Biology, Chemistry and Environmental Health Science, Benedict College, Columbia, SC 29204, USA
[c] Department of Pathology, Microbiology and Immunology, University of South Carolina School of Medicine, Columbia, SC, USA
[d] Division of Gastroenterology, Duke University, Durham, NC 27707, USA

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ABSTRACT

High circulatory insulin and leptin followed by underlying inflammation are often ascribed to the ectopic manifestations in non-alcoholic fatty liver disease (NAFLD) but the exact molecular pathways remain unclear. We have shown previously that CYP2E1-mediated oxidative stress and circulating leptin in NAFLD is associated with renal disease severity. Extending the studies, we hypothesized that high circulatory leptin in NAFLD causes renal mesangial cell activation and tubular inflammation via a NOX2 dependent pathway that upregulates proinflammatory miR21. High-fat diet (60% kcal) was used to induce fatty liver phenotype with parallel insulin and leptin resistance. The kidneys were probed for mesangial cell activation and tubular inflammation that showed accelerated NASH phenotype and oxidative stress in the liver. Results showed that NAFLD kidneys had significant increases in α-SMA, a marker of mesangial cell activation, miR21 levels, tyrosine nitration and renal inflammation while they were significantly decreased in leptin and p47 phox knockout mice. Micro RNA21 knockout mice showed decreased tubular immunotoxicity and proinflammatory mediator release. Mechanistically, use of NOX2 siRNA or apocynin,phenyl boronic acid (FBA), DMPO or miR21 antagomir inhibited leptin primed-miR21-mediated mesangial cell activation in vitro suggesting a direct role of leptin-mediated NOX-2 in miR21-mediated mesangial cell activation. Finally, JAK-STAT inhibitor completely abrogated the mesangial cell activation in leptin-primed cells suggesting that leptin signaling in the mesangial cells depended on the JAK-STAT pathway. Taken together the study reports a novel mechanistic pathway of leptin-mediated renal inflammation that is dependent on NOX-2-miR21 axis in ectopic manifestations underlying NAFLD-induced co-morbidities.

1. Introduction

Fatty liver is the most common cause of chronic liver injury [1]. Non-alcoholic fatty liver disease (NAFLD) is well-defined as the excessive accumulation of fat (> 5%) in the liver without excessive consumption of alcohol and often considered as a benign disease in the background of altered mediators of metabolic syndrome and pro-inflammatory immune response [1,2]. If untreated, NAFLD can progress from simple steatosis to complex stage of nonalcoholic steatohepatitis (NASH), hepatic fibrosis and hepatocellular carcinoma [3] following a second or multiple hits from oxidative stress, underlying low grade sterile inflammation or environmental factors [4]. The contribution of NAFLD/NASH in several ectopic diseases and pathological conditions such as type 2 diabetes (T2DM), cardiovascular disease (CVD), Chronic Kidney Disease (CKD) has been discussed in previous studies [5,6].

Recently, enormous interest has been generated following multiple

* Correspondence to: Environmental Health and Disease Laboratory, Department of Environmental Health Sciences, University of South Carolina, Columbia 29208, USA.
E-mail address: schatt@mailbox.sc.edu (S. Chatterjee).
[1] Author contributed equally.

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studies dealing with NAFLD/NASH-associated CKD. [7]. We have shown previously that accelerated glomerular pathology follows a NASH phenotype [8]. Additionally, CKD represents a major health concern in adults over age 65 years and it covers 25% of the western populations [9]. Commonly, CKD is described as decreased estimated glomerular filtration (eGFR) and/or increased proteinuria. At the advanced stage, CKD patients develop the end-stage renal disease because of the high risk of cardiovascular disease. Further, NAFLD is characterized by metabolic disturbances including insulin resistance and leptin resistance and inflammatory response in the liver. Leptin is a cytokine mainly produced by adipocytes and plays a proinflammatory role in the liver [10,11]. The primary function of leptin is to regulate satiety and control fat metabolism [12]. The increased levels of circulatory leptin (hyperleptinemia) found in NAFLD can influence inflammatory response in an ectopic organ such as the kidney in addition to other co-morbidities already reported in the clinics [7,13,14]. In the advanced stage of NAFLD/NASH, the common factor associated with NAFLD/NASH progression such as oxidative stress, activations of JAK/STAT, TGF-β signaling, renin-angiotensin system, TLR4 pathways and release of inflammatory cytokine may strongly associate with kidney disease [8,15–19].

We and others have previously shown that oxidative stress is the key regulator in NASH via second hit/multiple hit theory [10,20–22]. The redox stress generated by xenobiotic enzyme Cytochrome p450 2E1 (CYP2E1) via a free-radical mechanism generates reactive oxygen species (ROS) and reactive nitrative species (RNS) in the liver [23]. The oxidative stress resulted from reductive metabolism of CYP2E1, can accelerate metabolic disturbances, leptin release and trigger host innate immune response (ref). The cascade of redox signaling, presence of damage associated molecular patterns (DAMPS) and presence of high circulatory leptin can stimulate NADPH oxidase system in distal organs such as the kidney [10,24,25]. The NADPH oxidase (NOX) is commonly expressed in both phagocytic and non-phagocytic cells [26]. In Kidney, NADPH oxidases have divergent localization in the renal cells which include mesangial cells, tubular cells endothelial cells and podocytes [27]. Though NOX4 is predominant in the kidney, NOX2 is primarily expressed on mesangial cells and podocytes, but their functional significance remains unclear [28]. The NOX2 is primarily composed of several subunits mainly GP91 phox (membrane subunits) and P47 phox (cytosolic subunit)[26]. When the proper signal stimulates NOX2 activation it leads to the alignment of the cytosolic subunit (p47phox) to the membrane subunit (gp91phox). Localized NOX2 is involved in superoxide generation and can lead to the increased burden of oxidative stress and kidney inflammation [29]. NOX2 has also been shown to activate the TLR4 system which includes recruitment of TLR4 into lipid rafts and further receptor dimerization and activation in several inflammatory responses [16,30]. Previously, we and others have shown that NOX2, when stimulated by leptin, can generate peroxynitrite in different disease model such as intestinal inflammation or inflammatory bowel disease (IBD), liver inflammation in NASH, [16,25,31]. Since evidence is scarce to explain the specific NOX2 mediator involved in TLR4 activation in the mesangial cell, we chose to investigate the role of peroxynitrite in NOX2 mediated TLR4 activation in kidney inflammation. Recently, it has been shown that the highly reactive oxidant, peroxynitrite mediates glomerular lesion by JAK/STAT signaling pathway [32]. In our previous studies, Leptin has been shown to stimulate epigenetic regulation and microRNA induction in experimental NASH [33]. The Non-coding microRNA (miR) negatively regulates target protein by degrading mRNA (transcript degradation) and/or interfering in the process of protein translation. miR21 has been significantly upregulated in several inflammatory responses such as experimental NASH, hematopoietic cells, and allergic response [33–35]. In both liver and kidney injury, miR21 plays a key role in fibroblast activation and is a prominent regulatory factor in Smad7/TGF-beta signaling [15,36,37]. Considering these facts, in the present study, we hypothesize that increased circulatory leptin due to CYP2E1-induced oxidative stress in NAFLD activates mesangial cells and renal inflammatory response. Further leptin, acting via JAK/STAT-mediated NOX2 activation in the glomerulus induces miR21-mediated proinflammatory pathway that affects the progression of kidney disease. This present study uses an established invivo mouse model of CYP2E1-primed NASH, leptin knockout (KO) mice, p47 phox KO mice and miR21 KO mice along with kidney mesangial cells to show the mechanism of NOX2-initiated renal inflammation.

2. Materials and methods

Bromodichloromethane (BDCM), a known substrate for CYP2E1-mediated oxidative stress in the liver and corn oil were purchased from Sigma-Aldrich (St. Louis, MO). Anti–α-SMA, anti–IL-1β, anti–TNF-α, anti-TLR-4, Anti-3-Nitrotyrosine (3-NT), anti-P47phox and anti-GP91phox primary antibodies were purchased from Abcam (Cambridge, MA). Species-specific biotinylated conjugated secondary antibody and streptavidin -HRP were purchased from Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA). Wild-type and gene-specific KO mice were purchased from The Jackson Laboratories (Bar Harbor, ME). NOX2-siRNA, RNAiMAX-Lipofectamine, Fluorescence-conjugated (Alexa fluor) secondary antibodies and ProLong Gold antifade mounting media with DAPI were bought from Thermo Fisher Scientific (Waltham, MA). Animal diets were purchased from Research Diets (New Brunswick, NJ). All other chemicals were purchased from Sigma Aldrich unless otherwise specified. Paraffinized tissue sections on slides were done by IRF, University of South Carolina School of Medicine and AML laboratories (Baltimore, MD).

2.1. Cell culture

Mouse kidney Mesangial cell line (CRL-1927) was purchased from ATCC (Manassas, VA) and maintained in Dulbecco's modified eagles medium, Corning (Tewksbury, MA). The media was supplemented with 10% fetal bovine serum, Atlanta Biologicals (Norcross, GA), 2 mM glutamine, 100 U/ml Penicillin, and 100 μg/ml streptomycin; Gibco (Grand Island, NY). Cells were serum-starved (DMEM with 0.25% FBS) and incubated overnight at 37 °C in a humidified atmosphere of 5% CO_2 before any treatment. The cells were then treated with recombinant mouse Leptin 100 ng/ml, Biovision (Milpitas, CA), miR21 inhibitor 20 μM (Qiagen, Valencia, CA), JAK-STAT inhibitor (Ruxolitinib) 10 μM (Invivogen, San Diego, CA), Apocynin 100 μM as an inhibitor of NADPH oxidase activity, Phenyl Boronic Acid (FBA) 100 μM as an scavenger for peroxynitrite, Spin trap DMPO 100 μM (5,5 dimethyl-1- Pyrroline N-oxide) (Alexis biochemical, San Diego, CA) as an scavenger of free radicals via spin trapping used either separately (respective control groups) or in combination with Leptin (treated groups) for 24 h at 37 °C in a humidified atmosphere of 5% CO_2. Upon completion of the experiment, cells were lysed in Trizol, Invitrogen (Grand Island, NY) for mRNA extraction. Another set of cells were plated on microscopic coverslips MatTek Corp, (Ashland, MA) and treated similarly. The adhered cells on coverslips were used for immune-fluorescence staining after completion of the treatment.

2.2. siRNA inhibition of NOX2

Actively growing mesangial cells were seeded into the 6 well plates at low density. At 60–70% confluency, the NOX2-siRNA transfection was done using Lipofectamine RNAiMAX transfection reagent in Opti-MEM media following manufacturer protocol (Invitrogen). Briefly, Lipofectamine RNAiMAX reagent was diluted in the Opti-MEM medium as recommended. Similarly, NOX2-siRNA (60 pmol) was diluted in Opti-MEM medium and it was added to the diluted lipofectamine RNAiMAX solution. Further, it was incubated at 37 °C incubator for 5 min. The siRNA-lipid complex formed was added to the cells in the final concentration of 25 pmol. The cells were then incubated for 48 h