Alteration in kidney glucose and amino acids are implicated in renal pathology in MRL/lpr mice

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Abstract

This study has employed high-resolution NMR spectroscopy of kidney extracts to study alterations in the concentrations of amino acids and glucose in systemic lupus erythematosus (SLE). We used the well-established mouse model of SLE, MRL/lpr, and their congenic controls, MRL/+. There was a substantial increase in the tissue concentration of branched-chain amino acids (133%), aromatic amino acids (134%) and glutathione (122%) in the lupus mice, compared to the controls. Since increased glucose can lead to fibrosis, we used [1-13C] glucose as a tracer to study its transport into the kidney. Significant increases in the levels of [1-13C] glucose (200% of controls) were observed in the MRL/lpr mice 15 min after its injection. 13C NMR spectra demonstrated that the 13C-label from [1-13C] glucose was not incorporated into glycolytic and Krebs cycle related metabolites within 15 min. Furthermore, we found that the expression of the profibrotic cytokine, TGFβ and the regulatory transcription factor Smad3 are significantly enhanced in MRL/lpr mice compared to the MRL/+ controls. The mRNA and protein expression of extracellular matrix proteins, fibronectin, laminin, and collagen IV were upregulated in the MRL/lpr mice compared to the controls. All these changes were significantly reduced by the complement (C) inhibitor, Crry. Our results suggest that C activation causes increased glucose concentration in the kidney, which can lead to the observed hyperglycemia. This may be one of the important factors that cause increased extracellular matrix (ECM) deposition through the TGFβ signaling in lupus mice and thereby lead to glomerulosclerosis that translates into increased kidney disease.

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1. Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disorder with a wide spectrum of disease features that affect different organs, including oftentimes the kidney. Manifestations include autoantibodies, abnormal immune complex processing, hypocomplementemia, and proliferative glomerulonephritis (GN). The MRL/MpJ-Tnfrsf6lpr (MRL/lpr) and the congenic MRL/MpJ+/+ (MRL/) mice are a well-studied lupus model as they demonstrate several comparable features observed in human patients [5,6,11–14]. The MRL/lpr mice have a Fas mutation and display features of autoimmunity and inflammation [12]. The MRL/+ mice do not have this mutation and therefore develop disease much later in life and therefore serve as appropriate controls [1,37]. Renal disease in MRL/lpr mice has similar features to SLE patients, with mesangial proliferative GN early in disease and diffuse proliferative and crescentic GN later in the course. Finally, glomerulosclerosis occurs in the terminal phase of disease that leads to renal failure and ultimately death.

Complement (C) activation plays a significant role in the pathogenesis of SLE. Complement cascade proceeds via the classical, alternative, or mannose-binding lectin pathways [35,36]. Activation through each of the three pathways converges at the formation of C3 convertase and leads to a cleavage of C3. The C3 fragment, C3b attaches covalently to the antigen–antibody complexes, which is followed by C5 binding and its cleavage to C5a and C5b. The generation of C5b begins the nonenzymatic assembly of the C5b-9 membrane attack complex, which can result in cellular death or activation after membrane insertion [22].
Previous studies have shown that complement inhibition with the C regulatory protein, Crry, protects kidney cells from C-mediated injury in vitro and experimental mouse models of nephrotoxic serum nephritis (NSN) and lupus from renal disease in vivo [3,27]. Crry is a membrane-bound rodent complement regulator that inhibits the cleavage of C3 into its active metabolites and blocks both the classic and the alternative pathway of complement activation [18,21,38].

The complement cascades and glucose pathways are closely intertwined. Complement was shown to participate in glucose homeostasis while glucose could influence the expression of complement regulators [24]. Alteration in glucose levels can cause glomerular mesangial cell proliferation and increased matrix synthesis [28,39]. Hyperglycemia activates the Smad protein family of transcription factors. Smad-2 and -3 activate TGFβ while Smad-6 and -7 inhibit TGFβ [31,40]. Furthermore, high glucose activates ERK and PKC-delta leading to enhanced TGFβ signaling [16]. TGFβ is the key mediator of fibrosis of kidney diseases in both experimental mouse models and patients. Glomerulosclerosis occurs in this lupus model due to increased extracellular matrix accumulation which is prevented by C inhibition [4] but the intracellular signaling remains to be elucidated.

This study was designed to determine the metabolic turnover in kidneys of MRL/lpr mice and the MRL/+ controls by nuclear magnetic resonance (NMR) spectroscopy. 13C NMR allows the simultaneous calculation of metabolite concentrations and metabolic fluxes and thereby tracks cellular metabolism and intercellular trafficking of substrates in a comprehensive and non-invasive manner. Inhibition of C activation prevented alteration in the downstream intracellular signaling pathways leading to increased ECM synthesis and subsequently organ failure. Our study suggests a correlation between the concentration of glucose and metabolites in kidney with ECM deposition and provides an insight into a possible mechanism of renal injury in lupus nephritis.

2. Materials and methods

2.1. Mice

To evaluate the role of complement in lupus, MRL/lpr and MRL/+ mice generated in the laboratory overexpressing Crry directed by the metallothionein-I promoter [2,26] were used. MRL/+ mice (Jackson Laboratories, Bar Harbor, ME) served as appropriate controls. In all animals, the presence of the Crry transgene was documented by polymerase chain reaction (PCR) and soluble Crry in sera was identified by enzyme-linked immunosorbent assay (ELISA). At 19 weeks of age the mice were killed for tissue harvest (≥10 per group). The mice were given food and water ad libitum and maintained in 12-h light and dark cycles. These studies were approved by the University of Chicago Animal Care and Use Committee.

2.2. Administration of [1-13C] glucose

24-Week-old MRL/lpr and MRL/+ mice were administered [1-13C]-glucose (200 mg/kg, intraperitoneally; Cambridge Isotope Laboratories, Andover, MA) and killed exactly 15 min later by decapitation. The blood was taken from the neck wound for the NMR-spectroscopic analysis of plasma glucose levels and fractional 13C enrichments ([1-13C]/[12C] glucose). Plasma glucose levels and the percentage 13C enrichment in glucose were found to be not significantly different between both groups (data not shown).

2.3. Preparation of kidney extracts

Immediately after sacrifice, blood samples were mixed with 20% perchloric acid (PCA) and stored at –80 °C. Kidneys were snap frozen in liquid nitrogen, powdered over liquid nitrogen, and homogenized in 8 ml of a 12% PCA solution at 0 °C using a motor-driven, polished glass tube Teflon pestle homogenizer. The homogenate and the blood samples were centrifuged at 40,000×g for 15 min; the supernatants were placed in an ice bath and neutralized to pH 7.0 with KOH. The precipitated KClO4 was sedimented by centrifugation (40,000×g for 15 min), and supernatants were lyophilized.

2.4. NMR spectroscopy

The lyophilized PCA extracts of kidney tissue and blood plasma were redissolved in 0.6 ml deuterium oxide (D2O) and centrifuged. The pH was adjusted to 7.0 with deuterium chloride and NaOD. 13C NMR spectra were recorded on Bruker DRX 600 or AMX 360 spectrometers, operating at frequencies of 600 MHz or 360 MHz for 1H and 150.9 MHz or 90.5 MHz for 13C measurements. 1H NMR spectra were recorded with a 5-mm H,C,N inverse triple resonance probe, 400 accumulations, repetition time of 15 s, spectral width 7183 Hz (DRX 600) or 3623 Hz (AMX 360), data size 16 K, zero filling to 32 K. Chemical shifts were referenced to lactate at 1.33 ppm. 13C NMR spectra were recorded with the AMX 360 spectrometer using a 5-mm Q,N,P probe, 20,000 accumulations, repetition time of 2.5 s, composite pulse decoupling with WALTZ-16, spectral width 20,833 Hz, data size 32 K (16 K), zero filling to 64 K (32 K). Chemical shifts were referenced to the glucose 1β signal at 96.8 ppm.

2.5. Quantitation of metabolite concentrations

The concentrations (μmol/g tissue) of metabolites were determined from fully relaxed 1H NMR spectra of kidney extracts, using (trimethylsilyl) propionic-2,2,3,3,4-acid as an external standard.

2.6. Kidney water measurement

The percent water content of the kidney was measured by heating the tissue to 200 °C for 72 h. The percentage of water in the tissue was calculated as (wet wt – dry wt) × 100/wet wt. Six measurements each were made for lupus and control mice, and values were arithmetically averaged.

2.7. Immunofluorescence (IF) staining

Kidney (4 μm) sections were fixed using ethanol:ether (1:1) and 95% ethanol in sequence. After three washes in PBS, the sections were incubated with antibodies specific to collagen IV, fibronectin, and laminin (Santa Cruz, CA) followed by fluorescein-conjugated anti-rabbit IgG (ICN Biomedicals, Inc., OH, USA) and viewed by IF microscopy. No staining was observed in control slides treated with non-immune rabbit IgG followed by FITC-labeled anti-rabbit antibody. Sections were scored using a Zeiss microscope at 400×. The slides were scored in a blinded fashion from 0 to 4 (0 indicating no staining and 4 the most intense staining).

2.8. Real-time PCR (qRT-PCR)

Frozen kidney (n = 5 in each group) was homogenized in Trizol reagent (Life Technologies BRL, Grand Island, NY), and total RNA was purified according to the manufacturer’s instructions. cDNA was generated from RNA using random hexamers as primers with the SuperScript first-strand synthesis kit (Life Technologies BRL), according to the manufacturer’s instructions. qPCR was performed using the Prism 7700 reactor and the SybrGreen intercalating dye method with HotStar DNA polymerase (Applied Biosystems, Foster City, CA). Each reaction was conducted in a total volume of 50 μl with primers at 200 nM, 1 mM dNTP, 3 mM MgCl2, and 10 μl of sample or standard cDNA. PCR was carried out with a hot start at 95 °C (5 min) followed by 45 cycles at 95 °C (15 s)/
2.9. Western blotting

Kidneys were immediately frozen in liquid nitrogen, powdered, and then homogenized on ice in PBS, pH 7.2, containing 10 mM EDTA, 10 mM iodoacetamide, 5 mM diisopropylfluorophosphate and 1% NP-40. Nuclei and cytoplasmic debris were pelleted at 14,000×g. The supernatant by BCA assay. Equal amounts of solubilized kidney proteins were dissolved in reducing sample buffer and electrophoresed through a 7.5% SDS-PAGE gel. The membranes were incubated in 0.1 mM NaCl, 0.02 mM Tris (pH 7.2), 0.5% Tween 20 (TBST) overnight, the membrane (Millipore, Bedford, MA, USA). After blocking in 5% non-fat milk, the membrane was incubated in horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (Sigma Aldrich, St. Louis, MO, USA) at a 1:3000 dilution in TBST for 1.5 h. Thereafter, the supernatants were dissolved in reducing sample buffer and electrophoresed through a 7.5% SDS-PAGE gel. The membranes were incubated in anti-mouse collagen IV, fibronectin, laminin, 5'-ATACGCGCTGA-GTGCGCTGCT-3'; 5'-ACCTGAAACA-ACTCATTATA-3'; 5'-TCGGCTATGAT-3'

Table 1

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3. Results

3.1. Changes in the concentrations of amino acids and glucose in kidneys of MRL/lpr mice

The concentrations of amino acids and glucose were calculated from 1H NMR spectra (Table 1). Fig. 1 shows segments of representative 1H NMR spectra of kidney extracts. The chemical shifts (ppm) were compared with previously published data. From the high-field region of these spectra (0.8–1.1 ppm) the concentrations of the three aromatic amino acids, valine, leucine and isoleucine were analyzed. The most abundant aromatic amino acids phenylalanine and tyrosine were calculated from their down-field resonances. For the quantification of glutathione, the proton resonance at C-4 of the glutamyl residue in glutathione was used. Due to PCA extraction, this resonance is the sum of the oxidized and reduced form of glutathione. Glucose concentrations were determined by the C1 resonance. In MRL/lpr mice, the kidney concentrations of these branched-chain and aromatic amino acids were increased to 128–137% of congeneric MRL/+ controls (p < 0.05). Glutathione increased to 122% of controls (p < 0.05). No significant alterations were observed for other amino acids, such as glutamate, glutamine, aspartate or alanine (data not shown). Most significant changes were observed for glucose levels, which increased to 148% of controls (p < 0.001) (Fig. 2).

3.2. Glucose transport into kidney is increased in lupus mice

Increased concentrations of glucose in MRL/lpr kidney suggest increased transport or decreased utilization. Therefore, their measured levels may be either from increased uptake or decreased glucose utilization, or even a combination of the two. We injected [1-13C] glucose for 15 min. Fig. 1 shows representative 13C NMR spectra of kidney extracts. In congeneric MRL/+ controls, the concentrations of [1-13C] glucose (calculated from the 13C satellites in 1H NMR spectra) were very low (7.93±0.52 nmol/g wet weight (ww)); all other signals in the 13C NMR spectra were due to naturally abundant 13C in glucose and metabolites (1.1%). However, in MRL/lpr mice, [1-13C] glucose accumulated 2-fold over controls to concentrations of 15.93±2.31 nmol/g ww without incorporation of 13C label into glycolytic- or Krebs cycle-related metabolites. On the other hand, plasma glucose levels and the percentage 13C-enrichments in glucose did not change significantly (data not shown).

3.3. Water content is altered in kidneys of MRL/lpr mice

The water content of kidneys was altered significantly in MRL/lpr mice as compared to the MRL/+ controls. Total water content in lupus kidney was increased 6% compared to the controls (Fig. 3).
3.4. Complement inhibition prevents the increased expression of the profibrotic signaling molecules TGFβ, protein kinase C-delta, Smad3 and Smad7 in MRL/lpr mice

High glucose is known to induce the expression of ECM proteins. Consistent with the glomerulosclerosis observed in kidneys of MRL/lpr mice, there was increased expression of ECM proteins. Since TGFβ induces synthesis of ECM proteins, collagen and fibronectin, in human mesangial cells, we studied the expression of this profibrotic cytokine and signaling molecules Smad-3 and -7 in kidneys of lupus mice and the role of C in this signaling pathway. TGFβ and Smad-3 mRNA expression were significantly increased in MRL/lpr mice as compared to their control, MRL/+ mice; this increase was significantly reduced by C inhibition, in mice with the Crry transgene (Fig. 4). In contrast, mRNA expression of Smad-7 was significantly reduced in MRL/lpr mice. Complement inhibition reduced this change, although it did not reach statistical significance.

3.5. Increased mRNA and protein expression of ECM proteins in lupus mice

A substantial increase in mRNA expression for fibronectin, laminin and collagen 4 was observed in MRL/lpr mice compared to the MRL/+ controls, as measured by quantitative RT-PCR (Fig. 5). This increase was prevented by complement inhibition in the Crry-tg MRL/lpr mice. Furthermore, this alteration in ECM mRNA expression was translated into altered protein expression as observed by both Western blotting and IF (Fig. 6a and b, respectively).

4. Discussion

SLE is an inflammatory, systemic, autoimmune disease. Lupus nephritis, with inflammation and scarring within the kidney, is a major cause of morbidity and mortality [7,8]. Increased glucose concentrations were observed in SLE patients due to lower glucose disappearance rates which correlated with the inflammatory indices in those studies [23]. In order to obtain an insight into alterations of metabolites and glucose in the kidneys of MRL/lpr mice, we used high-resolution NMR spectroscopy and glucose labeled with the stable isotope 13C ([1-13C] glucose) [41]. Our results indicate a stimulated glucose transport in kidney in MRL/lpr mice compared to their congenic MRL/+ controls.

About 60% of adult patients with lupus nephritis have renal tubular dysfunction that includes distal renal tubular acidosis, impaired tubular potassium excretion, hyporenine-
mic hypoaldosteronism, and decreased urinary concentrating ability [9,17,19]. Renal gluconeogenesis plays an important role in conditions where acidosis occurs such as diabetes and gradually replaces hepatic gluconeogenesis. Normally, glucose produced from glutamate provides up to 4% of circulating plasma glucose in vivo [16]. But under these conditions, glucose produced by the kidney can account for a significantly higher percentage of circulating plasma glucose [32–34].

Kidneys play an important role in amino acid homeostasis. The concentrations of both branched-chain and aromatic amino acids were altered in kidneys of MRL/lpr mice. The alterations in the amino acid concentrations indicate decreased protein synthesis or increased protein degradation or both. The kidney is a dynamic organ, rich in the enzymatic machinery required for amino acid catabolism and/or oxidation, in particular in the medullary thick ascending limb, which provides energy to sustain active ion transport [10]. Therefore an alteration in amino acid levels suggests changes in ion transport and points to the value of future investigations of amino acid metabolism and ion transport.

Increased glomerular antioxidant enzymes were observed in a wide variety of glomerular diseases including lupus nephritis, indicating increased oxidative stress in these settings. In the present study, glutathione, an important antioxidant, was increased in lupus mice, which might suggest increased GSH formation as an adaptive response to oxidative stress in these animals. Cysteine is the rate-limiting substrate in de novo synthesis of GSH. In human vascular smooth muscle cells, the uptake rate of cystine decreased with increasing glucose concentrations, and supplying cysteine in the form of lipoic acid resulted in restoration of GSH [25]. Cysteine remained unaltered in the present study, suggesting that there was no change in its availability, and the increase in GSH was a protective response to the existing oxidative stress.

In the present study, ~6% increase in kidney water content was observed in the MRL/lpr mice as compared to their controls. Urea content in the kidney was also increased about 2-fold (results not shown). Glucose is an osmolyte, which along with increased urea, branched-chain and aromatic amino acids could contribute to the increased water content in the kidneys of the diseased mice.

Increased concentration of glucose could occur due to increased transport into the kidney or by decreased utilization. The results obtained using [1-13C] glucose demonstrate a 2-fold stimulated transport of glucose into the kidney, while this substrate is not metabolized via glycolytic or Krebs cycle activity by the kidney within 15 min. Our results suggest that increased transport could result in increased glucose levels in kidney, which could be one of the key facilitators inducing increased ECM protein expression. Given that glucose transport has been shown to induce fibronectin and collagen expression [15], this is an attractive explanation for all these findings.

High medium glucose concentration stimulates increased expression of ECM constituents such as collagen, fibronectin, and laminin by cells in culture [7]. Glomerulosclerosis that occurs in lupus includes mesangial expansion and accumulated ECM. We therefore studied ECM accumulation and the
associated signaling molecules in this lupus model. Similar to earlier studies, the expression of the profibrotic cytokine, TGFβ, was significantly induced in MRL/lpr mice [17]. Since glucose levels were increased in the kidney we studied the intracellular signaling pathway observed in hyperglycemia. TGFβ modulates ECM expression in mesangial cells via interaction with Smads [29,30]. Smad-3 expression (which is an important signal mediator involved in the fibrotic signal pathway) was increased and Smad-7 expression was decreased in MRL/lpr mice strongly suggesting for the first time that the ECM synthesis in lupus nephritis may follow the same signaling pathway induced by increased glucose concentration.

Increased glucose alters the constitutive mRNA expression of C components. C activation is an important facet of the disease in lupus. Earlier studies have shown that C activation is definitely linked to enhanced ECM synthesis and renal fibrosis [20]. The signaling pathway through which this occurs remains to be deciphered. Our results in this study demonstrate that the increased transport of glucose, the subsequent signaling and the increased ECM accumulation were all reduced by C inhibition giving us an insight into the possible mechanism for renal fibrosis in lupus.

In summary, our approach using high-resolution NMR spectroscopy provides evidence for the first time that renal glucose concentration and some aspects of kidney metabolism, e.g., amino acid metabolism, are altered in lupus mice and points towards future research in the direction of ion homeostasis. Further, our study suggests that C regulated glucose alteration and the subsequent signaling cascade could cause glomerulosclerosis and ultimately result in organ failure observed in lupus.

Fig. 6. (a) Proteins isolated from kidneys of MRL/lpr and MRL/lpr+Cry-tg were subjected to Western blotting technique using antibodies against collagen 4, fibronectin, laminin. In each lane proteins from a different mouse were loaded. Given here are 3 representative lanes from each group. There were 5 animals in each group. Values given below each sample are intensity of the band normalized to the corresponding actin intensity analyzed by Image J program. (b) Complement inhibition with Cry reduces the accumulations of collagens IV, fibronectin and laminin in 24-week-old MRL/lpr mouse glomeruli. Shown are representative immunofluorescence micrographs from 24-week-old MRL/+ mice (A), MRL/lpr mice treated with saline (B) and MRL/lpr mice with Cry transgene (C), and stained for collagen IV, fibronectin and laminin. In MRL/lpr mice there are increased deposits of the three ECM proteins collagen 4, fibronectin and laminin. Arrows indicate staining in a sclerotic mesangial area. ECM protein expression in MRL/lpr mice was reduced by complement inhibition with Cry. Pictures were taken using a Zeiss microscope at 40× magnification.
Acknowledgements

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References


