ORIGINAL RESEARCH

Association of Lipopolysaccharide-Toll-Like Receptor 4 Signaling and Microalbuminuria in Patients with Type 2 Diabetes Mellitus

Lijuan Zhang[1],*, Yuanjun Zhang[2,3],*, Juxiang Liu[2,3],*, Yonghong Li[4], Jinxing Quan [2,3]

[1]Department of Blood Transfusion, Gansu Provincial Hospital, Lanzhou, People's Republic of China; [2]Department of Endocrinology, Gansu Provincial Hospital, Lanzhou, People's Republic of China; [3]Key Laboratory of Endocrine and Metabolic Diseases of Gansu Province, Lanzhou, People's Republic of China; [4]Institute of Clinical and Translational Medicine, Gansu Provincial Hospital, Lanzhou, People's Republic of China

*These authors contributed equally to this work

Correspondence: Jinxing Quan, Tel +18109440427, Email quanjxxt@163.com

Purpose: Intestinal flora imbalance has been implicated in the activation of innate immunity in the kidneys. However, little is known about the potential links between lipopolysaccharide (LPS)-toll-like. receptor 4 (TLR4) signaling activated by intestinal barrier dysfunction and microalbuminuria in type 2 diabetes mellitus (T2DM).

Patients and Methods: 61 patients with T2DM were stratified based on the absence (n=32) or presence (n=29) of microalbuminuria. There were also 28 control subjects. Urinary albumin excretion rate (UAER), serum levels of LPS, D-lactic acid (DLA), diamine oxidase (DAO), fasting blood glucose (FBG), interleukin-6 (IL-6), glycosylated hemoglobin A1 (HbA1c), and high-sensitivity C-reactive protein (hs-CRP), and TLR4 expression in peripheral blood mononuclear cells (PBMCs) were measured.

Results: hs-CRP, IL-6, LPS, DLA, DAO, and TLR4 were markedly increased in subjects with T2DM compared to the controls (P < 0.05 for all). Moreover, LPS was positively correlated with FBG, HbA1c, hs-CRP, IL-6, UAER, DLA, DAO, and TLR4 (P < 0.05 for all). In addition, TLR4 was positively correlated with UAER, hs-CRP, FBG, DLA, HbA1c, and LPS (P < 0.05 for all). In regression analyses, TLR4, LPS, HbA1c, and hs-CRP were independently associated with UAER (P < 0.05 for all), while FBG, LPS, TLR4, and hs-CRP (P < 0.05 for all) were found to be risk factors for microalbuminuria in T2DM.

Conclusion: Intestinal integrity is compromised in subjects with T2DM, and the activation of LPS-TLR4 signaling might play an important role in the development of microalbuminuria in T2DM.

Keywords: intestinal mucosal barrier, microalbuminuria, type 2 diabetes mellitus, lipopolysaccharide, toll-like receptor 4

Introduction

Diabetic nephropathy (DN) is the leading cause of end-stage renal failure worldwide.[1,2] Several factors are involved in the pathophysiology of DN, including hyperglycemia, oxidative stress, and activation of the renin–angiotensin system.[3,4] However, surprisingly, strict control of these factors cannot completely prevent the progression of DN. Therefore, innovative exploration of the pathogenesis of early DN is crucial.

Intestinal flora disturbance is a new research area to explore in relation to developing therapeutic strategies for DN. The intestinal epithelial monolayer serves as a vital barrier between the organism and its surroundings. Normally, the intestinal barrier permits water, nutrients, and bioactive molecules to flow from the lumen to the bloodstream while preventing the translocation of harmful substances such as microbial and food-related antigens.[5] Diabetic and obese patients are prone to intestinal flora disturbance.

Gut dysbiosis is characterized by a predominance of LPS-enriched Gram-negative intestinal bacteria and a reduced level of butyrate-producing bacteria and other bacteria necessary for gut integrity.[6] This increases the translocation of LPS, D-lactic acid (DLA) and diamine oxidase (DAO) across the intestinal barrier into the bloodstream.[7–10] TLR4,

serves as a signaling receptor for LPS,[11,12] and its activation by LPS results in release of critical pro-inflammatory cytokines, such as interleukin (IL)-6 and C-reactive protein (CRP).[13] Studies in rodents have shown that LPS-TLR4 signaling induces an innate immune response that mediate systemic inflammation and insulin resistance.[14]

DN is a microvascular complication of diabetes, and the pathophysiology of DN is related to the presence of a chronic low-grade inflammatory state.[15] Recent research demonstrated that an intestinal flora imbalance may contribute to low-grade inflammation, leading to insulin resistance and T2DM.[16,17]

In recent years, circulating endotoxins have been implicated in the activation of innate immunity in the kidneys. Nymark et al reported that high LPS level in blood is linked to type 1 diabetic nephropathy.[18] Verzola et al showed that TLR4 gene and protein expression and TLR4 downstream signaling were markedly upregulated in biopsied kidney tissue from T2DM patients with DN.[19] These findings highlight the pivotal role of innate immunity in the development and progression of DN. However, little is known about the potential links between the LPS-TLR4 signaling activated by intestinal barrier dysfunction and DN in T2DM, including when low levels of albumin in the urine (microalbuminuria) develops, which represents the earliest stage of DN.

To address this issue, we aimed to investigate the serum LPS level, DLA level, and DAO activity, along with TLR4 expression in peripheral blood mononuclear cells (PBMCs) and systemic levels of IL-6 and high-sensitivity C-reactive protein (hs-CRP) in T2DM patients with or without microalbuminuria. Our results may provide new insights into the inhibition of signaling pathways as a potential treatment for DN patients.

Materials and Methods

Patient Selection

The research was carried out at Gansu Provincial Hospital's Department of Endocrinology. The Gansu Provincial Hospital ethics committee approved the study protocol and the study was performed according to the Declaration of Helsinki. All patients provided written informed consent before partaking in the research. A total of 61 patients with T2DM and 28 control subjects were prospectively recruited between April 2020 and June 2021. After a 5-min rest, both systolic and diastolic pressure were measured twice (with a 10-min interval) using a standardized mercury sphygmo-manometer, and the mean values were recorded. T2DM was diagnosed using the World Health Organization's (WHO's) 2006 diabetes diagnosis criteria[20] and microalbuminuria was diagnosed based on the presence of a urinary albumin excretion rate (UAER) of 20–200 μg/min in the absence of hematuria or infection.[21] The T2DM patients were categorized into two groups based on the value of UAER: T2DM+microalbuminuria group (UAER=20–200 μg/min; n=29) and T2DM group (UAER <20 μg/min; n=32). Members of the control group were recruited from our hospital's Physical Medical Examination Center.

Refusal to participate, active cancer, hypertension, gastrointestinal pathologies (such as peptic ulcer, irritable bowel syndrome, chronic gastroenteritis, chronic diarrhea, ulcerative colitis, or Crohn's disease), diabetes-related acute complications, inflammatory diseases (such as periodontitis or pneumonia), chronic hepatic or renal dysfunction, infectious diseases, history of gastrointestinal surgery, and treatment in the past 3 months with thiazolidinedione, biguanides, α-glucosidase inhibitors, statins, angiotensin-converting enzyme inhibitors, vitamin D, or antioxidants were the exclusion criteria.

Biochemical Measurements

Following an overnight fast (at least 12 hours), blood was drawn from all subjects in tubes with no anticoagulant. After 30 minutes of congealing, the blood was centrifuged for 10 minutes at 3000g. Serum samples were kept in Eppendorf tubes at −80°C until the tests were run. In the evening before the assays, To defrost the serum samples, they were kept at 4°C. Using an automatic biochemical analyzer (AU5400, Olympus, Beckman Coulter, USA), the researchers evaluated serum levels of triglycerides (TG), creatinine clearance (Ccr), high-density lipoprotein cholesterol (HDL), fasting blood glucose (FBG), total cholesterol (TC), creatinine (Cr), and low-density lipoprotein cholesterol (LDL). A semi-automated high-performance liquid chromatography system was used to determine HbA1c levels (HLC-723G7, Tosoh, Shanghai, China). A chemiluminescence immunoassay was used to assess the levels of fasting insulin in the blood (Abbott,

Chicago, USA).The formula for calculating the HOMA-IR (homeostatic model assessment of insulin resistance) index is: HOMA-IR = fasting serum insulin (mU/L) × FBG (mmol/L)/22.5. Each patient's urine was collected for 24 hours and stored at 2–8°C until analysis to determine the UAER. An immunoturbidimetric test was used to detect the urine albumin level (DCA 2000 Analyzer, Bayer, Germany) and then UAER (μg/min) was computed as: UAER = total urinary albumin (μg) in 24-h urine/1440min. The within and between run coefficient of variation (CV) was <5%.

Flow Cytometric Analysis of TLR4 in PBMCs

TLR4 expression in PBMCs was detected using flow cytometry. Briefly, 100 μL ethylenediaminetetraacetic acid (EDTA)-anticoagulated peripheral whole blood was incubated with 5 μL mouse anti-human TLR4 monoclonal antibody (eBioscience, California, USA) and 10 μL mouse anti-human CD14 monoclonal antibody (BD, New Jersey, USA) for 30 min in the dark at room temperature, followed by incubation with 500 μL red blood cell lysis buffer for 10 min. After washing twice with phosphate-buffered saline (PBS), the cell pellets were resuspended in 500 μL PBS and then analyzed with a FACSCalibur flow cytometer (BD) to assess the TLR4 expression. A tube without fluorescent antibodies was used as the blank control. Cell Quest software (BD) was used to analyze the data, and the percentage of double-positive cells stained with antibodies against CD14 and TLR4 was recorded.

Serum DLA, DAO, LPS, IL-6, and hs-CRP Measurements

Competitive Enzyme-Linked Immunosorbent Assay (ELISA) for Serum DAO Activity, DLA Level, and LPS Level (Hermes Criterion Biotechnology Inc., Vancouver, Canada). The minimum detectable concentrations for DLA, DAO, and LPS were 0.1 μg/mL, 1.0 ng/mL, and 1.0 pg/mL, respectively. The intra- and inter-assay CV values were <8% and <12%, respectively.

Serum IL-6 was measured by electrochemiluminescence (Roche Diagnostics GmbH, Mannheim, Germany). The minimum detectable concentration for IL-6 was 1.5 pg/mL. The intra- and inter-assay CV values were <7% and <13%, respectively. Serum hs-CRP was measured by an immunoturbidimetric method (Jiuqiang Biotechnology Institute, Beijing, China). The minimum detectable concentration for hs-CRP was 0.015 mg/dL. The intra- and inter-assay CV values were <5% and <10%, respectively. All samples were assessed twice.

Data Analysis

SPSS version 19.0 (SPSS Inc., Chicago, IL, USA) was used to conduct all data analysis. Continuous variables with a normal distribution are presented as mean ± standard deviation (SD) and were compared by one-way analysis of variance (ANOVA). Continuous variables with a non-normal distribution are presented as median (interquartile range) and were compared by the Kruskal–Wallis for samples. Categorical variables are presented as n (%) and were compared by the chi-square test. The relationships between variables were also assessed using Spearman correlation analysis. Multivariate stepwise linear regression analysis was used to evaluate the factors that were independently associated with UAER in T2DM. Binary logistic regression analyses were used to evaluate the risk factors for microalbuminuria in T2DM. Two-sided P<0.05 indicated statistical significance.

Results
Clinical and Biochemical Characteristics

T2DM patients (mean age: 50.41 ± 10.38 years; N = 32), T2DM patients with microalbuminuria (mean age: 54.20 ± 11.74 years; N = 29), and control subjects (mean age: 51.86 ± 5.39 years; N = 28) were included in this study. There were no significant differences in sex distribution, blood pressure, BMI, TC, TG, or smoking and alcohol consumption among the three groups. The levels of LPS, DLA, DAO, hs-CRP, TLR4 expression, FBG, HbA1c, HOMA-IR, urinary albumin, and UAER were significantly increased in both the T2DM group and the T2DM+microalbuminuria group compared to the control group (P < 0.05 for all). These factors were more elevated in the T2DM+microalbuminuria group than in the T2DM group (P < 0.05 for all). The serum IL-6 level was markedly higher in the T2DM+microalbuminuria group than the other two groups (P < 0.05), but there was no difference between the T2DM and control groups. Lastly, the HDL level was significantly lower in both the T2DM and T2DM +microalbuminuria group compared to the control group (P < 0.05 for all) (Table 1 and Figure 1).

**Table 1** Clinical and Laboratory Characteristics of the Participants

Parameter	Control Group (n = 28)	T2DM Group (n = 32)	T2DM+Microalbuminuria Group (n = 29)	P value
Age (years)	51.86±5.39	50.41±10.38	58.38±11.89 [c]	0.005
Sex (male, %)	14 (50.00%)	19 (59.38%)	16 (55.17%)	0.767
T2DM duration (years)	–	1.50 (0.10, 7.75)	8.00 (2.00, 13.50) [c]	0.007
Smoking, n (%)	5 (17.86%)	10 (31.25%)	5 (17.24%)	0.331
Alcohol consumption, n (%)	7 (25.00%)	13 (40.62%)	8 (27.59%)	0.370
BMI (kg/m^2)	23.72±2.44	24.65±3.32	24.68±3.05	0.384
SBP (mmHg)	120.25±12.60	125.69±16.02	125.10±25.72	0.489
DBP (mmHg)	76.79±5.92	79.06±9.80	79.69±8.80	0.395
FBG (mmol/L)	5.35±0.81	10.27±3.41 [b]	11.54±3.83 [b, c]	<0.001
HbA1c (%)	5.75±0.31	9.99±2.02 [b]	10.14±2.36 [b, c]	<0.001
HOMA-IR	1.42 (0.97, 2.29)	3.12 (1.76, 4.14) [b]	3.23 (1.97, 4.42) [b, c]	<0.001
TC (mmol/L)	4.79±0.77	5.04±0.84	4.53±1.15	0.118
TG (mmol/L)	1.28 (1.00, 1.61)	1.74 (1.22, 2.59)	1.43 (1.19, 2.11)	0.044
HDL (mmol/L)	1.48±0.55	1.19±0.24 [b]	1.16±0.22 [b]	0.002
LDL (mmol/L)	3.07±0.78	3.65±0.77 [b]	3.17±1.10	0.027
Cr (μmol/L)	66.00±13.70	56.66±11.90	61.65±15.97	0.036
Ccr (mL/min)	123.98±40.96	111.31±32.84	114.86±41.70	<0.001
Urinary albumin (mg/24h)	2.17 (0.82,12.30)	10.61 (3.63, 15.08) [b]	65.52 (40.39, 154.18) [b, c]	<0.001
UAER (μg/min)	1.51 (0.57, 9.48)	7.36 (2.52, 10.48) [b]	45.50 (29.04, 107.07) [b, c]	<0.001
TLR4 (%)	10.48±2.10	12.72±3.34 [b]	14.23±5.25 [b, c]	0.001
IL-6 (pg/mL)	4.00 (3.33, 5.48)	3.30 (2.73, 4.20)	5.90 (4.15, 9.25) [b, c]	<0.001
hs-CRP (mg/dL)	0.35 (0.20, 0.70)	0.85 (0.20, 1.50) [b]	2.30 (0.75, 3.95) [b, c]	<0.001
DLA (ug/mL)	3.44±0.97	4.52±1.66 [b]	5.02±1.83 [b, c]	0.001
DAO (ng/mL)	231.28±49.89	267.32±79.83 [b]	299.99±67.55 [b, c]	0.001
LPS (pg/mL)	583.26±198.56	748.40±339.94 [b]	1212.47±422.15 [b, c]	<0.001

Notes: All values are expressed as mean ± SD, median value (interquartile range), or n (%); [b]P < 0.05 vs control group; [c] P < 0.05 vs T2DM group.
Abbreviations: T"DM, type 2 diabetes mellitus; Cr, creatinine; UAER, urinary albumin excretion rate; BMI, body mass index; SBP, systolic blood pressure; DAO, diamine oxidase; DBP, diastolic blood pressure; FBG, fasting blood glucose; HbA1c, glycosylated hemoglobin A1c; LPS, lipopolysaccharide; HOMA-IR, homoeostasis model assessment-insulin resistance; TC, cholesterol; TLR4, toll-like receptor 4; TG, triglyceride; HDL, high-density lipoprotein; LDL, low-density lipoprotein; Ccr: creatinine clearance; IL-6, interleukin-6; DLA, d-lactic acid; hs-CRP, high-sensitivity C-reactive protein.

Correlation Analyses

Spearman correlation analyses showed that both LPS and UAER were positively correlated with T2DM duration, FBG, DAO, HOMA-IR, HbA1c, hs-CRP, IL-6, DLA, and TLR4, while being negatively correlated with HDL (Tables 2 and 3); notably, LPS was positively correlated with UAER. TLR4 was positively correlated with T2DM duration, FBG, HOMA-IR, HbA1c, hs-CRP, UAER, DLA, and LPS, but had no correlation with age, HDL, IL-6, or DAO levels (Table 4).
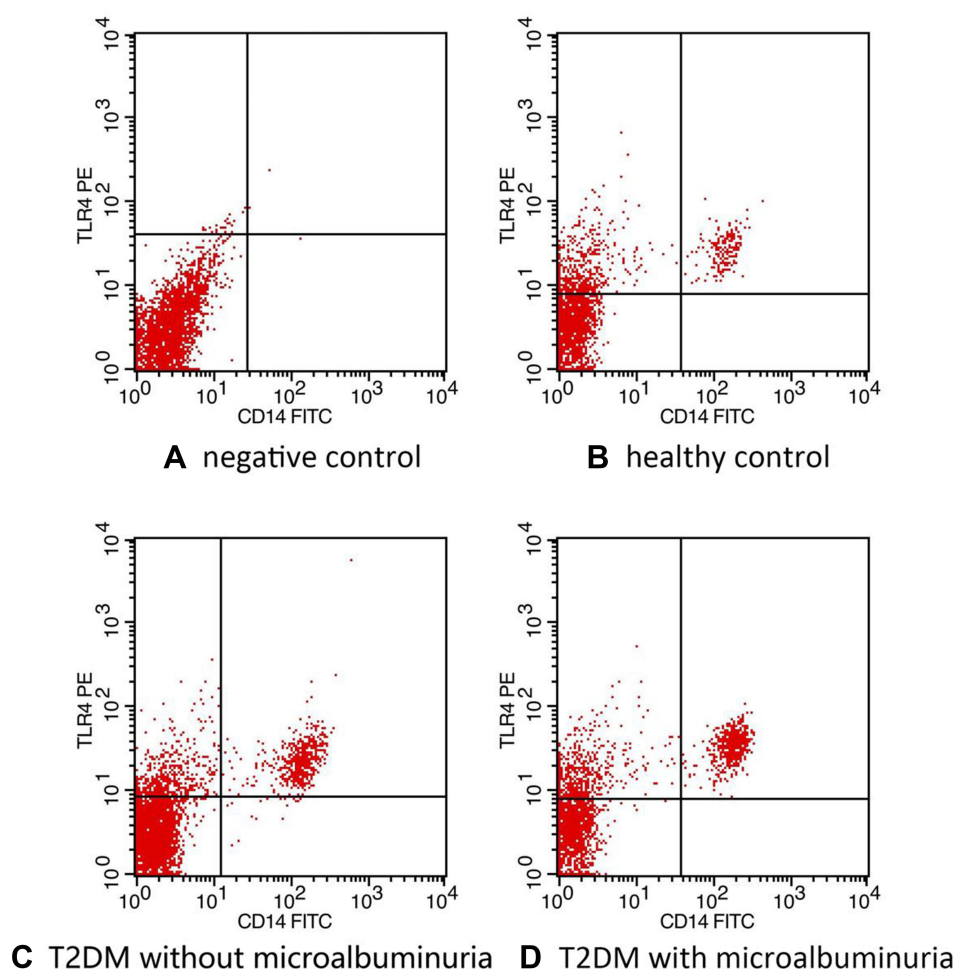
Figure 1 TLR4 expression in peripheral blood mononuclear cells (PBMCs) (**A**) negative control, (**B**) control, (**C**) T2DM, and (**D**) T2DM+microalbuminuria groups. The percentage of double-positive cells stained with antibodies against CD14 and TLR4 was assessed by flow cytometry. The blank control was a tube without fluorescent antibodies.

Regression Analyses

Multivariate stepwise linear regression analysis showed that TLR4, LPS, HbA1c and hs-CRP were significantly independently associated with UAER (Table 5). Binary logistic regression analysis showed that FBG (odds ratio [OR] = 1.415, 95% CI = 1.045–1.918), LPS (OR = 1.012, 95% CI = 1.004–1.018), TLR4 (OR = 1.254, 95% CI = 1.010–1.557), and hs-CRP (OR = 1.984, 95% CI = 1.264–3.122) were significant risk factors for microalbuminuria in T2DM (Table 6).

Discussion

Emerging evidence has demonstrated that the inflammatory milieu in T2DM significantly contributes to the development of the vascular complications of T2DM, including DN. However, little is known about the association between chronic low-grade inflammation induced by LPS-TLR4 signaling and the development of DN in T2DM.

Table 2 Correlations of Serum LPS with Clinical and Laboratory Characteristics

	Age	Duration	FBG	HOMA-IR	HbA1c	HDL	hs-CRP	IL-6	UAER	DLA	DAO	TLR4
rho	0.242	0.480	0.420	0.380	0.372	−0.214	0.281	0.248	0.556	0.594	0.663	0.251
P value	0.022	0.000	0.000	0.000	0.000	0.044	0.008	0.019	0.000	0.000	0.000	0.018

Abbreviations: LPS, lipopolysaccharide; FBG, fasting blood glucose; UAER, urinary albumin excretion rate; HbA1c, glycosylated hemoglobin A1c; HOMA-IR, homoeostasis model assessment-insulin resistance; DLA, d-lactic acid; HDL, high-density lipoprotein; TLR4, toll-like receptor 4; IL-6, interleukin 6; DAO, diamine oxidase; hs-CRP, high-sensitivity C-reactive protein.

Table 3 Correlations of UAER with Clinical and Laboratory Characteristics

	Age	Duration	FBG	HOMA-IR	HbA1c	HDL	hs-CRP	IL-6	LPS	DLA	DAO	TLR4
rho	0.193	0.597	0.481	0.271	0.450	−0.279	0.333	0.322	0.556	0.270	0.294	0.291
P value	0.070	0.000	0.000	0.010	0.000	0.008	0.001	0.002	0.000	0.010	0.005	0.006

Abbreviations: UAER, urinary albumin excretion rate; TLR4, toll-like receptor 4; LPS, lipopolysaccharide; FBG, fasting blood glucose; hs-CRP, high-sensitivity C-reactive protein; HDL, high-density lipoprotein; HbA1c, glycosylated hemoglobin A1c; DLA, d-lactic acid; HOMA-IR, homoeostasis model assessment-insulin resistance; IL-6, interleukin-6; DAO, diamine oxidase.

Table 4 Correlations of TLR4 Expression with Clinical and Laboratory Characteristics

	Age	Duration	FBG	HOMA-IR	HbA1c	HDL	hs-CRP	IL-6	UAER	LPS	DLA	DAO
rho	0.120	0.334	0.229	0.231	0.224	−0.012	0.272	0.041	0.291	0.251	0.220	0.020
P value	0.261	0.001	0.031	0.029	0.035	0.909	0.010	0.706	0.006	0.018	0.039	0.853

Abbreviations: TLR4, toll-like receptor 4; LPS, lipopolysaccharide; FBG, fasting blood glucose; hs-CRP, high-sensitivity C-reactive protein; HbA1c, glycosylated hemoglobin A1c; DAO, diamine oxidase; HOMA-IR, homoeostasis model assessment-insulin resistance; DLA, d-lactic acid; HDL, high-density lipoprotein; UAER, urinary albumin excretion rate; IL-6, interleukin-6.

Table 5 Multivariate Stepwise Linear Regression Analysis of Factors Independently Associated with UAER

Parameter	β	95% CI	t	P value
LPS	0.041	0.010—0.072	2.651	0.010
HbA1c	5.419	0.046—10.792	2.008	0.048
TLR4	2.401	0.097—4.705	2.074	0.041
hs-CRP	6.211	1.355—11.068	2.546	0.013

Abbreviations: UAER, urinary albumin excretion rate; LPS, lipopolysaccharide; HbA1c, glycosylated hemoglobin A1c; TLR4, toll-like receptor-4; DLA, d-lactic acid; hs-CRP, high-sensitivity C-reactive protein; CI, confidence interval.

Table 6 Binary Logistic Regression Analysis of Risk Factors for Microalbuminuria in T2DM

Parameter	Odds Ratio	95% CI	P value
FBG	1.415	1.045–1.918	0.024
hs-CRP	1.984	1.264–3.122	0.003
TLR4	1.254	1.010–1.557	0.041
LPS	1.012	1.004–1.018	0.000

Abbreviations: T2DM, type 2 diabetes mellitus; DLA, d-lactic acid; FBG, fasting blood glucose; TLR4, toll-like receptor 4; CI, confidence interval; hs-CRP, high-sensitivity C-reactive protein; LPS, lipopolysaccharide.

DLA is a product of gut bacteria anaerobic glycolysis, and DAO is a highly active intracellular enzyme in intestinal villi. Both are released into the blood when the intestinal mucosa is damaged.[22] In this study, DLA and DAO, both of which are indicative of intestinal mucosal barrier function,[22,23] were used to evaluate the permeability of the intestine. Serum DLA and DAO were increased in subjects with T2DM compared to controls. Serum LPS was also significantly elevated in subjects with T2DM compared to controls. These findings are in accordance with previous reports describing

the occurrence of intestinal barrier dysfunction in both mice[14,24] and humans[25,26] with T2DM, suggesting that intestinal integrity is compromised in subjects with T2DM.

Additionally, serum DLA, DAO, and LPS were positively correlated with UAER (Table 3), indicating that intestinal flora imbalance may be related to albuminuria in T2DM. Relatedly, previous studies have shown that intestinal flora disturbance accelerates disease progression in DN patients.[27,28] He et al showed that the gut microbiota composition and function was altered in T2DM patients with DN compared to those without DN (based on metagenomic sequencing of fecal samples),[29] but the exact pathogenetic mechanism remains unclear.

It is noteworthy that in the present study, serum LPS was more elevated in T2DM patients with microalbuminuria than in those with T2DM alone and LPS was positively correlated with UAER, indicating that LPS may play a role in the development of albuminuria in T2DM. Relatedly, a previous study reported that LPS promotes the development of DN in T1DM mice.[18,30] Similarly, Nymark et al described an association between LPS activity and the development of microalbuminuria in Finnish patients with T1DM.[18] We did not identify any other studies on the association between the serum LPS level and microalbuminuria. For the first time, we show that increased serum LPS levels are associated with microalbuminuria in T2DM. Furthermore, LPS was positively correlated with metabolic parameters such as HbA1c, HOMA-IR, and FBG. These findings are consistent with previous reports describing the role played by LPS in insulin resistance, glycemic regulation, and lipid metabolism.[24,31]

The mechanisms involved in this endotoxemia state are probably related to both increased LPS uptake and decreased LPS clearance. The chylomicrons secreted by enterocytes take up more LPS, and defective tight junctions between the enterocytes leads to increased intestinal permeability (known as "leaky gut"). This ultimately causes more LPS to translocate or leak into the blood circulation.[16,17,32,33] Concurrently, hyperglycemia impairs macrophage function and other monocyte function while suppressing leukocyte bactericidal activity,[34,35] thereby increasing LPS production. In addition, HDL is considered to be the most important element in LPS detoxification or neutralization.[36] In our study, LPS was negatively correlated with serum HDL and positively correlated with FBG and HbA1c. This indicates that reduced HDL and impaired neutrophil function due to hyperglycemia may decrease LPS clearance. Together, these mechanisms may worsen the endotoxemia, which may reduce T2DM control and contribute to related vascular complications.

TLR4 is a pivotal factor in innate immunity and inflammation and is expressed on macrophages/monocytes as well as nonimmune cells, such as native kidney cells.[37] Upon stimulation, TLR4 activates the nuclear factor (NF)-kB pathway, inducing the production of proinflammatory cytokines and triggering inflammatory responses. It has been reported that in the presence of a high glucose concentration in monocytes, TLR4 is elevated in vitro,[38] and elevated TLR4 expression has been observed in monocytes from T2DM patients.[39] Our study is the first to demonstrate that TLR4 signaling is activated in PBMCs from T2DM patients with microalbuminuria, which was manifested as increased TLR4 expression in human PBMCs and elevated serum levels of IL-6 and CRP in T2DM patients with microalbuminuria compared to T2DM patients and controls. Similarly, a previous study observed that T2DM patients with DN and uremia exhibited increased TLR4 expression in PBMCs, along with elevated serum LPS, IL-6, and hs-CRP.[40]

Given that uremia can damage the intestinal barrier and thereby increase LPS levels,[41] it is difficult to conclude the causative relationship between elevated LPS levels and macroalbuminuria in this setting. Of note, in our study, LPS was positively correlated with TLR4 and its downstream cytokines IL-6 and hs-CRP, both of which have been implicated in the development of albuminuria in T2DM.[42,43] Moreover, multivariate linear regression showed that LPS and TLR4 were independently associated with UAER and binary logistic regression showed that they were risk factors for microalbuminuria in T2DM patients. Collectively, these findings suggest that the activation of LPS-TLR4 signaling plays an important role in the development of microalbuminuria in T2DM. Zhao et al reported that Tangshen formula in DN rats modulated the gut microbiota, inhibited LPS production, and downregulated TLR4, which reduced renal inflammation,[44] which supports our findings based on research on humans.

Regarding the mechanisms governing how activated LPS-TLR4 signaling exerts its role in the pathogenesis of DN in T2DM, several possible explanations should be noted. First, LPS promotes the development and progression of DN in T1DM.[18] Additionally, TLR4 and its downstream inflammatory cytokines (such as IL-6) are highly expressed in kidney tissue obtained from biopsies of T2DM patients,[19] and TLR4 knockdown prevents DN fibrosis and podocyte injury in T1DM mice.[45] Accordingly, it is likely that increased circulating LPS activates TLR4 directly in kidney macrophages

and triggers inflammatory responses, leading to kidney impairment. Second, monocytes/macrophages are the principal inflammatory cells in diabetic kidneys, and recent studies have suggested that monocytes/macrophages play an important role in the pathogenesis of glomerulopathy and tubulointerstitial lesions in DN.[19,46,47] In animal models of DN in T1DM, kidney macrophage accumulation was associated with albuminuria and renal fibrosis.[45] It is therefore plausible that kidney infiltration of circulating activated monocytes by LPS-TLR4 signaling may cause kidney damage. Finally, it is noteworthy that in addition to gut-derived endotoxins, hyperglycemia and free fatty acids are also involved in the pathogenesis of DN in T2DM through activation of LPS-TLR4 in PBMCs and kidneys.[47] We found that there were increased circulatory levels of LPS and TLR4 expression in PBMCs in T2DM patients with microalbuminuria compared to the T2DM and control groups, which expands our understanding of the emerging role of the gut microbiota in the pathogenesis of DN. This is likely to be of clinical significance because current therapeutic options, which includedstrict blood glucose and blood pressure management, as well as renin-angiotensin system blockade, are unable to entirely prevent DN progression. Targeting the intestinal microbiota to modulate the intestinal barrier dysfunction and reduce serum levels of LPS in T2DM seems to be a promising approach to treat DN, particularly at the microalbuminuria stage, because DN seems to be reversible at this stage. Interestingly, several probiotics and prebiotics can improve glucose tolerance in mice withT2DM and improve insulin sensitivity in subjects without T2DM.[24,48] Whether these compounds also exert effective control over DN merits further research. Our study's biggest weakness is the limited sample size. There are relatively few T2DM subjects with microalbuminuria who are not taking antidiabetic agents, such as metformin, thiazolidinediones, and glycosidase inhibitors, all of which may affect LPS and TLR4 expression. This prohibited the study from including a wide range of patients and may have reduced the statistical power. The number of patients included in the study, however, was sufficient to statistically compare the various groups. In addition, because of the cross-sectional nature of this study, the causal relationships could not be determined between LPS and metabolic parameters such as glucose and insulin levels, which may interact with LPS-TLR4 signaling and contribute to the development of DN. Although DN is correlated with duration, different diabetes durations may have an impact on TLR4 or LPS. We have excluded the effect when modeling.Further large-scale prospective studies are required to obtain additional data.

Conclusion

Collectively, our findings suggest that the activation of LPS-TLR4 signaling plays an important role in the development of microalbuminuria in T2DM. Therefore, targeting the intestinal microbiota to modulate intestinal barrier dysfunction and reduce serum LPS in T2DM or blocking LPS-TLR4 signaling are promising approaches to treat DN.

Acknowledgments

Funding

Disclosure

The authors report no conflicts of interest in this work.

References

1. Stanton RC. Diabetic Kidney Disease and Hypertension. *Exp Clin Endocrinology Diabetes*. 2016;124(2):93–98. doi:10.1055/s-0035-1559760
2. Maqbool M, Cooper ME, Jandeleit-Dahm KAM. Cardiovascular Disease and Diabetic Kidney Disease. *Semin Nephrol*. 2018;38(3):217–232. doi:10.1016/j.semnephrol.2018.02.003
3. Wada J, Makino H. Inflammation and the pathogenesis of diabetic nephropathy. *Clin Sci*. 2013;124(3):139–152. doi:10.1042/cs20120198
4. Navarro-González JF, Mora-Fernández C, Muros de Fuentes M, García-Pérez J. Inflammatory molecules and pathways in the pathogenesis of diabetic nephropathy. *Nat Rev Nephrol*. 2011;7(6):327–340. doi:10.1038/nrneph.2011.51

5. Patterson E, Ryan PM, Cryan JF, et al. Gut microbiota, obesity and diabetes. *Postgrad Med J*. 2016;92(1087):286–300. doi:10.1136/postgradmedj-2015-133285

6. Fava F, Gitau R, Griffin BA, Gibson GR, Tuohy KM, Lovegrove JA. The type and quantity of dietary fat and carbohydrate alter faecal microbiome and short-chain fatty acid excretion in a metabolic syndrome 'at-risk' population. *Int J Obes*. 2013;37(2):216–223. doi:10.1038/ijo.2012.33

7. Gomes AC, Bueno AA, de Souza RG, Mota JF. Gut microbiota, probiotics and diabetes. *Nutr J*. 2014;13:60. doi:10.1186/1475-2891-13-60

8. Chambers ES, Byrne CS, Morrison DJ, et al. Dietary supplementation with inulin-propionate ester or inulin improves insulin sensitivity in adults with overweight and obesity with distinct effects on the gut microbiota, plasma metabolome and systemic inflammatory responses: a randomised cross-over trial. *Gut*. 2019;68(8):1430–1438. doi:10.1136/gutjnl-2019-318424

9. Sun YJ, Cao HJ, Song DD, Diao YG, Zhou J, Zhang TZ. Probiotics can alleviate cardiopulmonary bypass-induced intestinal mucosa damage in rats. *Dig Dis Sci*. 2013;58(6):1528–1536. doi:10.1007/s10620-012-2546-0

10. Sun XQ, Fu XB, Zhang R, et al. Relationship between plasma D(-)-lactate and intestinal damage after severe injuries in rats. *World j Gastroenterol*. 2001;7(4):555–558. doi:10.3748/wjg.v7.i4.555

11. Beutler B. Tlr4: central component of the sole mammalian LPS sensor. *Curr Opin Immunol*. 2000;12(1):20–26. doi:10.1016/s0952-7915(99)00046-1

12. Miller SI, Ernst RK, Bader MW. LPS, TLR4 and infectious disease diversity. *Nat Rev Microbiol*. 2005;3(1):36–46. doi:10.1038/nrmicro1068

13. Tran AX, Whitfield C. *Lipopolysaccharides (Endotoxins). Encyclopedia of Microbiology*. 3rd ed. Oxford: Academic Press; 2009:p.513–528.

14. Cani PD, Bibiloni R, Knauf C, et al. Changes in gut microbiota control metabolic endotoxemia-induced inflammation in high-fat diet-induced obesity and diabetes in mice. *Diabetes*. 2008;57(6):1470–1481. doi:10.2337/db07-1403

15. Brownlee M. The pathobiology of diabetic complications: a unifying mechanism. *Diabetes*. 2005;54(6):1615–1625. doi:10.2337/diabetes.54.6.1615

16. Sohail MU, Althani A, Anwar H, Rizzi R, Marei HE. Role of the Gastrointestinal Tract Microbiome in the Pathophysiology of Diabetes Mellitus. *J Diabetes Res*. 2017;2017:9631435. doi:10.1155/2017/9631435

17. Gomes JMG, Costa JA, Alfenas RCG. Metabolic endotoxemia and diabetes mellitus: a systematic review. *Metabolism*. 2017;68:133–144. doi:10.1016/j.metabol.2016.12.009

18. Nymark M, Pussinen PJ, Tuomainen AM, Forsblom C, Groop PH, Lehto M. Serum lipopolysaccharide activity is associated with the progression of kidney disease in finnish patients with type 1 diabetes. *Diabetes Care*. 2009;32(9):1689–1693. doi:10.2337/dc09-0467

19. Verzola D, Cappuccino L, D'Amato E, et al. Enhanced glomerular Toll-like receptor 4 expression and signaling in patients with type 2 diabetic nephropathy and microalbuminuria. *Kidney Int*. 2014;86(6):1229–1243. doi:10.1038/ki.2014.116

20. Medicine IOC. *Definition and Diagnosis of Diabetes Mellitus and Intermediate Hyper-Glycemia*. Geneva World Health Organization; 2006.

21. Patel S, Thompson AN. Screening, diagnosis, and management of type II diabetes: a review of the 2014 American Diabetes Association guidelines. *Acta physiologica*. 2014;4(3):107–113.

22. Xu J, Liu Z, Zhan W, et al. Recombinant TsP53 modulates intestinal epithelial barrier integrity via upregulation of ZO-1 in LPS-induced septic mice. *Mol Med Rep*. 2018;17(1):1212–1218. doi:10.3892/mmr.2017.7946

23. Wu M, Xiao H, Ren W, et al. Therapeutic effects of glutamic acid in piglets challenged with deoxynivalenol. *PLoS One*. 2014;9(7):e100591. doi:10.1371/journal.pone.0100591

24. Cani PD, Neyrinck AM, Fava F, et al. Selective increases of bifidobacteria in gut microflora improve high-fat-diet-induced diabetes in mice through a mechanism associated with endotoxaemia. *Diabetologia*. 2007;50(11):2374–2383. doi:10.1007/s00125-007-0791-0

25. Creely SJ, McTernan PG, Kusminski CM, et al. Lipopolysaccharide activates an innate immune system response in human adipose tissue in obesity and type 2 diabetes. *Am J Physiol Endocrinol Metab*. 2007;292(3):E740–747. doi:10.1152/ajpendo.00302.2006

26. Brar PC, Kohn B. Use of the microbiome in the management of children with type 2 diabetes mellitus. *Curr Opin Pediatr*. 2019;31(4):524–530. doi:10.1097/mop.0000000000000781

27. Evenepoel P, Poesen R, Meijers B. The gut-kidney axis. *Pediatric Nephrol*. 2017;32(11):2005–2014. doi:10.1007/s00467-016-3527-x

28. Ramezani A, Raj DS. The gut microbiome, kidney disease, and targeted interventions. *J Am Soc Nephrol*. 2014;25(4):657–670. doi:10.1681/asn.2013080905

29. He X, Sun J, Liu C, et al. Compositional Alterations of Gut Microbiota in Patients with Diabetic Kidney Disease and Type 2 Diabetes Mellitus. *Diabetes Metab Syndr Obes*. 2022;15:755–765. doi:10.2147/dmso.S347805

30. Kajiwara K, Takata S, To TT, et al. The promotion of nephropathy by Porphyromonas gingivalis lipopolysaccharide via toll-like receptors. *Diabetol Metab Syndr*. 2017;9:73. doi:10.1186/s13098-017-0271-8

31. Lassenius MI, Pietiläinen KH, Kaartinen K, et al. Bacterial endotoxin activity in human serum is associated with dyslipidemia, insulin resistance, obesity, and chronic inflammation. *Diabetes Care*. 2011;34(8):1809–1815. doi:10.2337/dc10-2197

32. Harte AL, Varma MC, Tripathi G, et al. High fat intake leads to acute postprandial exposure to circulating endotoxin in type 2 diabetic subjects. *Diabetes Care*. 2012;35(2):375–382. doi:10.2337/dc11-1593

33. Zhou SY, Gillilland M, Wu X, et al. FODMAP diet modulates visceral nociception by lipopolysaccharide-mediated intestinal inflammation and barrier dysfunction. *J Clin Invest*. 2018;128(1):267–280. doi:10.1172/jci92390

34. Yu WK, Li WQ, Li N, Li JS. Influence of acute hyperglycemia in human sepsis on inflammatory cytokine and counterregulatory hormone concentrations. *World j Gastroenterol*. 2003;9(8):1824–1827. doi:10.3748/wjg.v9.i8.1824

35. Torres-Castro I, Arroyo-Camarena ÚD, Martínez-Reyes CP, et al. Human monocytes and macrophages undergo M1-type inflammatory polarization in response to high levels of glucose. *Immunol Lett*. 2016;176:81–89. doi:10.1016/j.imlet.2016.06.001

36. Wendel M, Paul R, Heller AR. Lipoproteins in inflammation and sepsis. II. Clinical aspects. *Intensive Care Med*. 2007;33(1):25–35. doi:10.1007/s00134-006-0433-x

37. Anders HJ. Toll-like receptors and danger signaling in kidney injury. *J Am Soc Nephrol*. 2010;21(8):1270–1274. doi:10.1681/asn.2010030233

38. Dasu MR, Devaraj S, Zhao L, Hwang DH, Jialal I. High glucose induces toll-like receptor expression in human monocytes: mechanism of activation. *Diabetes*. 2008;57(11):3090–3098. doi:10.2337/db08-0564

39. Dasu MR, Devaraj S, Park S, Jialal I. Increased toll-like receptor (TLR) activation and TLR ligands in recently diagnosed type 2 diabetic subjects. *Diabetes Care*. 2010;33(4):861–868. doi:10.2337/dc09-1799

40. Yang M, Gan H, Shen Q, Tang W, Du X, Chen D. Proinflammatory CD14+CD16+ monocytes are associated with microinflammation in patients with type 2 diabetes mellitus and diabetic nephropathy uremia. *Inflammation*. 2012;35(1):388–396. doi:10.1007/s10753-011-9374-9

41. Magnusson M, Magnusson KE, Sundqvist T, Denneberg T. Impaired intestinal barrier function measured by differently sized polyethylene glycols in patients with chronic renal failure. *Gut*. 1991;32(7):754–759. doi:10.1136/gut.32.7.754

42. Navarro-González JF, Mora-Fernández C. The role of inflammatory cytokines in diabetic nephropathy. *J Am Soc Nephrol*. 2008;19(3):433–442. doi:10.1681/asn.2007091048

43. Chuengsamarn S, Rattanamongkolgul S, Sittithumcharee G, Jirawatnotai S. Association of serum high-sensitivity C-reactive protein with metabolic control and diabetic chronic vascular complications in patients with type 2 diabetes. *Diabetes Metab Syndr*. 2017;11(2):103–108. doi:10.1016/j.dsx.2016.08.012

44. Zhao T, Zhang H, Yin X, et al. Tangshen formula modulates gut Microbiota and reduces gut-derived toxins in diabetic nephropathy rats. *Biomed Pharmacother*. 2020;129:110325. doi:10.1016/j.biopha.2020.110325

45. Jialal I, Major AM, Devaraj S. Global Toll-like receptor 4 knockout results in decreased renal inflammation, fibrosis and podocytopathy. *J Diabetes Complications*. 2014;28(6):755–761. doi:10.1016/j.jdiacomp.2014.07.003

46. Garibotto G, Carta A, Picciotto D, Viazzi F, Verzola D. Toll-like receptor-4 signaling mediates inflammation and tissue injury in diabetic nephropathy. *J Nephrol*. 2017;30(6):719–727. doi:10.1007/s40620-017-0432-8

47. Sabatino A, Regolisti G, Cosola C, Gesualdo L, Fiaccadori E. Intestinal Microbiota in Type 2 Diabetes and Chronic Kidney Disease. *Curr Diab Rep*. 2017;17(3):16. doi:10.1007/s11892-017-0841-z

48. He M, Shi B. Gut microbiota as a potential target of metabolic syndrome: the role of probiotics and prebiotics. *Cell Biosci*. 2017;7:54. doi:10.1186/s13578-017-0183-1