Table 4. Hazard ratios for the cardiovascular composite endpoint.

	Univariate model		Multivariate model [a]	
	Hazard ratio (95% CI)	***P* value**	**Adjusted Hazard ratio (95% CI)**	***P* value**
Age (year)	1.08 (1.05–1.11)	< 0.001	1.07 (1.04–1.11)	<0.001
Systolic BP (mmHg)	1.02 (1.01–1.04)	0.001	0.99 (0.99–1.02)	0.85
Hypertension (yes)	4.08 (2.07–8.05)	<0.001	2.06 (0.95–4.46)	0.07
Log HDL-cholesterol (mg/dl)	0.02 (0.01–0.26)	0.002	0.16 (0.01–2.00)	0.16
Log UAER (μg/min)	2.14 (1.60–2.92)	<0.001	1.56 (1.04–2.35)	0.03
eGFR (ml/min/1.73 m^2)	0.97 (0.96–0.99)	<0.001	1.00 (0.99–1.02)	0.63
baPWV (m/sec)	1.00 (1.00–1.01)	0.008	1.00 (0.99–1.00)	0.42
CVD-AI	4.62 (2.65–8.04)	<0.001	2.86 (1.57–5.19)	0.001

The variables listed in Table 1 and CVD-AI were firstly assessed in the univariate analysis of the Cox proportional hazards regression model. Only variables shown to be statistically significant in the univariate model are shown in this table.

[a]Each estimate was adjusted for all variables shown in this table.

Abbreviations: BP, blood pressure; CI, confidence interval, CVD-AI, cardiovascular disease-amino acid based index; HDL, high density lipoprotein; UAER, urinary albumin excretion rate; eGFR, estimated glomerular filtration rate; baPWV, brachial-ankle pulse wave velocity.

doi:10.1371/journal.pone.0101219.t004

UAER ($\rho = 0.30$, $P<0.001$), and inversely correlated with HDL-cholesterol ($\rho = -0.21$, $P<0.001$) and eGFR ($\rho = -0.39$, $P< 0.001$), although it was not correlated with HbA1c level ($\rho = 0.08$, $P = 0.12$). Furthermore, the CVD-AI values in patients with antihypertensive agents were higher than those without (-1.54 ± 0.88 *vs.* -1.97 ± 0.63, $P<0.001$), whereas the CVD-AI values were not different among the three patient subgroups stratified by antidiabetic medication (diet only, oral agents and insulin therapy).

Compared with the AUC for ROC curve analysis, the CVD-AI showed better discriminatory ability (0.72 [95% CI: 0.64–0.79]) than did the level of each amino acid (Table 3). Even when validated by LOOCV analysis, the AUC of the CVD-AI ROC was 0.68. ROC curve analysis showed that the CVD-AI cut-off level for this outcome was −1.662. In Cox proportional hazards regression analysis, patients with the CVD-AI above the cut-off level showed a significantly higher unadjusted HR of 4.62 (95% CI: 2.65–8.04) for the cardiovascular composite endpoint, as did age, systolic BP, hypertension, HDL, UAER, eGFR, and baPWV (Table 4). Even when adjusted for these variables shown to be statistically significant in the univariate model, the CVD-AI, as well as age and UAER, was identified as an independent risk for this outcome (adjusted HR: 2.86, [95% CI: 1.57–5.19], Table 4).

Next, we separately estimated the risk of the CVD-AI for two conditions: coronary vascular events (myocardial infarction and angina pectoris, n = 40) and cerebrovascular events (stroke, n = 18). Unadjusted HR for coronary vascular events was 5.51 (95% CI: 2.85–10.64). Adjusted for variables listed in Table 4, the risk of the CVD-AI for coronary vascular events did not change (adjusted HR: 3.35 [95% CI: 1.64–6.83]). In contrast, the unadjusted and adjusted HR for stroke were 2.61 (95% CI: 0.99–6.85) and 1.51 (95% CI: 0.52–4.37), respectively.

Combination effect of UAER and CVD-AI

In this study, UAER has also been identified as an independent risk for cardiovascular outcome, as in previous reports, and the AUC for ROC curve analysis of UAER (0.69 [95% CI: 0.62–0.77]) was almost equally to that of the CVD-AI (0.72 [95% CI: 0.64–0.79]). We thus finally analyzed the combination effect of UAER and CVD-AI in predicting cardiovascular composite endpoints. For this purpose, patients were divided into four subgroups: those with normoalbuminuria and above or below the cut-off level of CVD-AI and those with albuminuria and above or below the cut-off level of CVD-AI (Table 5). In patients with a CVD-AI above the cut-off level, both those with normoalbuminuria (unadjusted HR: 3.24 [95% CI: 1.54–6.82]) and albumin-

Table 5. Crude and multivariate-adjusted hazard ratios for the cardiovascular composite endpoint in patient subgroups stratified according to urinary albumin excretion rate and the CVD-AI.

Subgroup category	**Total (n)**	**Case (n)**	**Crude Hazard ratio (95% CI)**	***P* value**	**Adjusted Hazard ratio [a] (95% CI)**	***P* value**
UAER <20 μg/min + Low CVD-AI	192	13	1.00 (reference)		1.00 (reference)	
UAER <20 μg/min + High CVD-AI	72	15	3.24 (1.54–6.82)	0.002	2.61 (1.23–5.54)	0.012
UAER ≥20 μg/min + Low CVD-AI	54	6	1.76 (0.67–4.63)	0.25	1.48 (0.55–3.99)	0.44
UAER ≥20 μg/min + High CVD-AI	63	29	8.25 (4.28–15.9)	<0.001	4.52 (2.09–9.80)	<0.001

Subjects were categorized as being above or below a UAER of 20 μg/min and above or below the CVD-AI cut-off value of −1.662. Crude (unadjusted) and adjusted hazard ratios were calculated using Cox proportional hazards regression models.

[a]Estimates were adjusted for the conventional risk factors of cardiovascular disease, including age, sex, HbA1c, total cholesterol, triglyceride, high density lipoprotein cholesterol, estimated glomerular filtration rate, body mass index and hypertension.

Abbreviations: CVD-AI, cardiovascular disease-amino acid based index; UAER, urinary albumin excretion rate.

doi:10.1371/journal.pone.0101219.t005

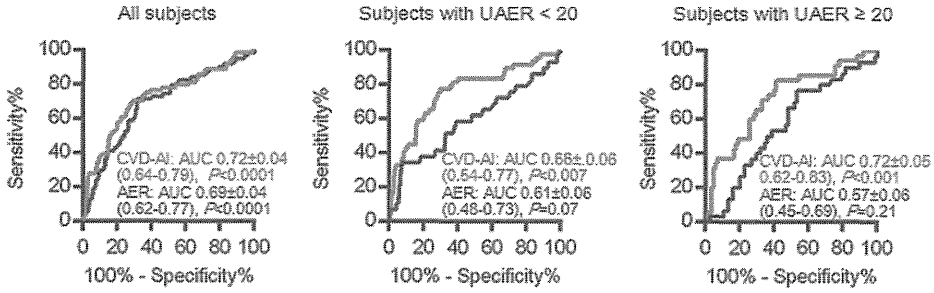

Figure 1. Results of area under the curve of receiver-operator characteristics curve analysis for both CVD-AI and urinary album excretion rate to distinguish cases from controls in all subjects and those with/without albuminuria.
doi:10.1371/journal.pone.0101219.g001

uria (unadjusted HR: 8.25 [95% CI: 4.28–15.9]) were at significantly higher risk for the onset of cardiovascular composite endpoints. Even after adjustment for the conventional risk factors of cardiovascular disease, both groups remained at risk (Table 5). In contrast, patients with a CVD-AI below the cut-off level, even those with albuminuria (HR: 1.48 [95% CI: 0.55–3.99]), were not at significant risk for this outcome.

We found that the CVD-AI could distinguish cases from controls even when patients with normoalbuminuria (AUC: 0.66, 95% CI: 0.54–0.77, $P=0.007$) and those with albuminuria (AUC: 0.72, 95% CI: 0.62–0.83, $P<0.001$) were separately analyzed (Figure 1). In contrast, UAER was unable to distinguish cases from controls, both in patients with normoalbuminuria (AUC: 0.61, 95% CI: 0.48–0.73, $P=0.07$) and those with albuminuria (AUC: 0.59, 95% CI: 0.46–0.69, $P=0.21$).

Discussion

Identification of a reliable surrogate marker or index for predicting the onset of CVD is essential in the care of patients with diabetes. Using high-throughput PFAA profiling and the data of our ongoing prospective observational follow-up study we constructed the diagnostic index, the CVD-AI, to predict the onset of CVD in patients with type 2 diabetes. Interestingly, this predictive effect was independent of the levels of albuminuria and the conventional risk factors of CVD, indicating that altered PFAA profiles were able to effectively identify high risk patients, even those without albuminuria. These findings suggest that the PFAA profile is a clinically useful index for improving the discriminative capability for coronary artery disease in diabetic patients in addition to conventional risk factors and better risk stratification even among those with normoalbuminuria, who are at relatively low risk for CVD.

Alterations in the composition of PFAAs have been reported to reflect the pathological status or preconditions in numerous diseases including CVD, suggesting that these alterations may be involved in disease development processes [18–21]. Several clinical studies using this new technology have reported on the association between the altered composition of PPFAs and the predictive effect for CVD. Shah *et al.* demonstrated that plasma metabolomic profiles, including several amino acids, have been found to predict cardiovascular events and improve risk discrimination beyond the degree possible using readily available clinical characteristics [20,21]. Magnusson *et al.* also reported that an amino acid index consisting of branched-chain and aromatic amino acids was found to strongly predict the development of CVD during 12 years of follow-up [19]. As with these previous reports, branched-chain amino acids and aromatic amino acids in the current study were found to correlate with obesity- and dyslipidemia-related risks for CVD. However, the predictive power of each amino acid for CVD was relatively weak, although some amino acids showed significantly different plasma levels between cases and controls. The CVD-AI based on the PFAA profiles, called "AminoIndex^{TM} technology" [11–13], improved the predictive effect for CVD in comparison to individual PFAAs. These results suggest that the CVD-AI is a more sensitive and effective predictive index than the conventional risk factors to identify patients at risk for CVD, although we need to validate the predictive effect of this CVD-AI.

The ability to identify patients at high risk of CVD before its onset is particularly important in diabetes care, because CVD can greatly affect mortality and quality of life in patients with diabetes. Albuminuria is a strong predictor for CVD, making the prevention of increased albuminuria and the reduction of albuminuria a therapeutic target for the prevention of CVD [2–7]. Although albuminuria was one of the risk factors for CVD in our population, as well as in previous reports, the CVD-AI showed almost equal or slightly better discriminatory capability than UAER in ROC curve analysis. In addition, the CVD-AI was identified as an independent risk factor for the onset of CVD even after adjusting the conventional risk factors including albuminuria in the Cox proportional hazards regression model. Interestingly, this predictive effect was observed even in patients with normoalbuminuria as well as those with albuminuria. Thus, PFAA profiles may be clinically useful as a novel index for identifying diabetic patients at high risk for CVD regardless of the degree of albuminuria or improving the discriminative capability by combining it with albuminuria.

It remains unclear whether the association between altered PFAA profiles and CVD onset represents a cause-effect relationship. Metabolic profiles have been reported to be highly heritable in families with early-onset CVD [22]. Thus, the susceptibility of diabetic patients to the onset of CVD may be due in part to genetically determined metabolic components. In this study, the CVD-AI significantly correlated with cardiovascular risk factors, particularly dyslipidemia, renal function and hypertension, whereas it did not correlate with HbA1c. This may mean that the CVD-AI reflects the influence of atherosclerosis rather than glycemic control. Also, amino acids are reported to directly contribute to insulin resistance by disrupting insulin signaling [23]. Because insulin resistance promotes the development of atherosclerosis, the altered PFAA profiles associated with insulin resistance may be indirectly associated with the onset of CVD. Unfortunately, we could not investigate the association between the CVD-AI and insulin resistance in this study. Further studies are needed to clarify whether the CVD-AI is a specific index for patients with diabetes mellitus.

This study had several limitations. This study was designed as a retrospective analysis of samples and data obtained during our prospective observational follow-up study, not as an interventional study. Thus, treatment protocols including dietary regimens were not controlled, and the influence of cofounders during the observational period was not analyzed. The time-dependent changes in PFAAs during follow-up periods were also not assessed. Therefore, it remains unclear as to whether the correction of these altered PFAA profiles represents a new therapeutic target to prevent CVD in patients with diabetes. Furthermore, we need to validate the CVD-AI using the PFAA profiles identified in this study, and further prospective studies are required to confirm whether our CVD-AI is most suitable for predicting the onset of CVD and to determine whether correcting the altered PFAA profiles can improve prognosis in patients with diabetes mellitus.

Conclusions

This study has demonstrated that altered PFAA profiles can predict the onset of CVD in patients with type 2 diabetes over a 10-year follow-up period. These alterations predicted the onset of CVD regardless of the degree of albuminuria and other conventional risk factors for CVD. Further prospective studies are required to validate the clinical utility of these PFAA measurements and to construct an optimal CVD-AI that can be used to identify diabetic patients at high risk for CVD in clinical practice.

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Acknowledgments

We are grateful to all members of the Maegawa Laboratory for their scientific input and contributions to the Shiga Prospective Observational Follow-up Study. We also thank Yumiko Omura and Keiko Kondo (Shiga University of Medical Science) for their excellent assistance.

Supporting Information

Table S1 The top ten models performance using ROC of AUC.
(PPTX)

Table S2. Correlation between plasma level of each amino acid and conventional cardiovascular risk.
(DOCX)

Author Contributions

Conceived and designed the experiments: SK SIA NO TU HM H. Maegawa. Performed the experiments: NO AS TM. Analyzed the data: SK SIA NO KN H. Miyano. Contributed reagents/materials/analysis tools: HA KI DK MH SU HK AK. Wrote the paper: SK SIA NO TU H. Kawai.

Original Article

Relationship between a Low Ankle Brachial Index and All-Cause Death and Cardiovascular Events in Subjects with and without Diabetes

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Aims: The association between a low ankle brachial index (ABI) and mortality and vascular morbidity in Japanese individuals with diabetes and the independence of this association from other risk factors have not yet been examined in the primary care setting among a large number of patients.
Methods: An observational prospective cohort study was performed among 3,004 Japanese individuals (2,598 patients with diabetes) to examine all-cause death and cardiovascular disease (CVD) in relation to low ABI (<0.9) values and other risk factors.
Results: Low ABI values were found in 127 subjects (4.2%) and was associated with smoking, diabetes, hypertension, pulse pressure, glycosylated hemoglobin A1c, lipid profiles, glomerular filtration rate, uric acid and prevalent CVD at baseline. Over 13,242 person-years, 93 deaths and 117 cases of CVD occurred. In a multivariate Cox regression analysis, the hazard ratio for low-normal ABI values was 3.97 (95% CI, 2.29 to 6.88) for all-cause death and 2.86 (95% CI, 1.83-4.49) for fatal and non-fatal CVD and all-cause death. Similar hazard ratios were found when the subjects were confined to those with diabetes. All risk analyses indicated that age, a low ABI, diabetes, a history of CVD and smoking remained significantly and independently predictive of CVD and all-cause death.
Conclusions: A low ABI exhibits significant cross-sectional associations with conventional risk factors and further more with the glomerular filtration rate, uric acid level and presence of prevalent CVD at baseline, and a low ABI independently predicts subsequent death and cardiovascular events. These findings support the concept that a low ABI is an integrated marker of an excess risk of death and cardiovascular events, independent of conventional risk factors.

J Atheroscler Thromb, 2014; 21:574-581.

Key words: Ankle brachial index, Cardiovascular disease, Diabetes, Primary care, Mortality

Introduction

The ankle brachial index (ABI) represents the ratio of the ankle to brachial systolic pressure. In patients with atherosclerotic stenosis in the lower extremities, the decreased pressure in the ankle arteries results in a lower ABI. The detection of a low ABI is useful for confirming the diagnosis and severity of peripheral artery disease (PAD) in the legs and is reportedly associated with an increased risk of all-cause death and cardiovascular disease (CVD) in the general population in Western countries[1-5]. The validity of a low ABI may be decreased in patients with diabetes, as the ankle pressure may be elevated due to medial arterial calcification and/or arterial stiffening, which occur more frequently in diabetes[6]. However, most prospective studies that have investigated the predictive value of a low ABI in patients with CVD

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Received: October 31, 2013
Accepted for publication: December 23, 2013

included less than 500 subjects with diabetes[3, 7-12], and some studies have indicated that the association between a low ABI and mortality in Caucasians is weak among subjects with diabetes compared to that observed in those without diabetes[5, 8, 13]. While ethnic differences profoundly affect the prevalence of PAD[10], no such prospective studies have been performed in Japanese subjects, who are characterized by a lower prevalence of PAD than Caucasian individuals[14-16]. A large-scale study of Japanese individuals including subjects with diabetes is needed to elucidate the association and predictive value of a low ABI with respect to the incidence of CVD.

In the primary care setting, screening for low ABI values is strongly recommended in subjects with diabetes, as these patients are often asymptomatic due to diabetic neuropathy, even when complicated with PAD[7, 17]. However, this recommendation has not been universally embraced, and measurement of the ABI is rarely applied in routine clinical practice[18]. We have obtained measurements of the ABI at the first visit to the clinic in routine clinical practice for more than 10 years. The present study investigated the cross-sectional associations between low ABI values and other cardiovascular risk factors at baseline and assessed the prognostic value of a low ABI for predicting death and cardiovascular events. This study included a large number of Japanese subjects with and without diabetes and explored whether the impact of a low ABI on outcomes is independent of other cardiovascular risk factors.

Subjects and Methods

Patient Recruitment

A prospective cohort study was performed to investigate the associations between low ABI values and cardiovascular risk factors at baseline and whether a low ABI is predictive of all-cause death and cardiovascular events independent of other risk factors. The health care system in Japan provides healthcare services with the patient accepting responsibility for 30% of the cost and the government paying the remaining 70%. Payment for personal medical services is offered through a universal health care insurance system that provides relative equality of access. Patients are free to select physicians or facilities of their choice. All consecutive patients 20 years of age or older who visited the outpatient clinic of Jiyugaoka Internal Medicine between 2001 and 2011 were enrolled in this study. The study was performed in a primary care setting. All of the subjects, most of whom had type 2 diabetes, hypertension or dyslipidemia, underwent ABI measurement at their first visit as a baseline routine examination (N=3501). Subjects who discontinued the visits within three months, primarily due to visiting other hospitals or moving to other cities, were excluded, leaving 3,004 subjects (non-diabetes: 406, type 2 diabetes: 2,572, type 1 diabetes: 26) eligible for this cohort. Those who discontinued visits were similar to the remaining patients with respect to clinical features. The study protocol was approved by the local ethics committee and carried out in accordance with the Helsinki Declaration II.

Baseline Examinations

A short physical examination and medical history assessment were performed at baseline in each patient. The presence of prevalent CVD at baseline included a history of coronary heart disease (CHD), cerebrovascular disease and/or PAD. The definition of CVD was the same as that described below. The smoking status was defined as current or not. Type 2 and type 1 diabetes was defined according to the Japan Diabetes Society criteria. Blood pressure (BP) was measured in the sitting position after a rest of more than five minutes. Hypertension was defined as a BP of ≥140/90 mmHg or the current use of antihypertensive agents. Non-fasting blood samples were obtained for measurements of the glycosylated hemoglobin A_{1c} level (HbA_{1c}, normal range: 4.6-6.2%) and serum creatinine (Cr), uric acid and lipid concentrations. Dyslipidemia was defined as a serum concentration of total cholesterol of ≥ 220 mg/dL, triglycerides of ≥ 150 mg/dL or high-density lipoprotein (HDL) cholesterol of <40 mg/dL and/or the current use of lipid-lowering agents. The non-HDL cholesterol level was calculated by subtracting the HDL cholesterol level from the total cholesterol level. The serum concentration of Cr was measured using an enzymatic method. The estimated glomerular filtration rate (eGFR) was calculated using the following equation proposed by the Japanese Society of Nephrology: eGFR (ml/min per 1.73 m^2) = $194 \times (\text{age [years]})^{-0.287} \times (\text{serum Cr [mg/dL]})^{-1.094} \times 0.739$ (if female).

The ABI was measured at baseline under standardized conditions. Doppler-assisted systolic blood pressure measurements were obtained from the brachial and posterior tibial arteries on both sides using 12-cm cuffs (Colin Co., Ltd., Komaki, Japan). The ABI was calculated for each leg using the highest ankle pressure divided by the highest systolic brachial pressure. An ABI of <0.9 in either leg was considered abnormal. Although an ABI of >1.4 has been indicated to be abnormally high as a result of poor arterial compressibility due to arterial stiffening and calcifica-

tion[3], there were only 16 subjects in this cohort; therefore, this parameter was not defined separately.

Main Outcomes

The subjects attended the clinic monthly and were followed from the baseline visit until the end of observation (August, 2012) or an event. Fatal and non-fatal CVD events included onset of coronary heart disease (CHD), ischemic cerebrovascular stroke and PAD. Information regarding the onset of cardiovascular events and the cause of death was provided by the medical doctors (e.g., cardiologists, neurologists and vascular surgeons) who managed the event. Three outcomes were assessed: 1) all-cause death, 2) the occurrence of cardiovascular events and 3) composite endpoints, including death and the occurrence of cardiovascular events.

Diagnosis of CVD

Non-fatal CHD included acute myocardial infarction with survival of more than 24 hours after the onset of symptoms, percutaneous coronary intervention, coronary artery bypass and new-onset unstable angina pectoris. A non-fatal ischemic stroke was defined as an acute focal neurological deficit lasting for longer than 24 hours. PAD was diagnosed in cases involving intermittent claudication with confirmation of an ABI of <0.9 or significant peripheral artery stenosis on angiography and/or leg amputation above the ankle due to diabetes. We classified sudden death as a cardiovascular event, unless there was a clear non-vascular cause. An independent panel, working with the endpoint adjudication committee, assessed all potential endpoints and classified them in accordance with the predefined criteria.

Statistical Analysis

The data are expressed as the mean ± SD, unless otherwise stated. For comparisons between two groups, unpaired Student's *t*-test, the Mann-Whitney U test for variables with a skewed distribution and the χ^2 test for categorical variables were used. A logistic regression analysis was used to assess the associations between the baseline risk factors and the concomitant presence of a low ABI following adjustment for the traditional cardiovascular risk factors of age, sex, BMI and smoking status. The follow-up time was calculated as the time between the baseline examination and either the date of the main outcome or the end of observation (December 31, 2011). For subjects who discontinued clinic attendance, the date of the final visit in cases in which no occurrence of events was confirmed was employed. The time to event distribution according to the ABI group was summarized with Kaplan-Meier curves. Cox regression models examining the effects of a low ABI on each event rate were adjusted for potential confounders. As to potential confounders, the conventional risk factors of age sex, BMI and smoking status were entered in the model, and diabetes, hypertension, dyslipidemia, eGFR and a past history of CVD were additionally considered. P-values under 5% (two-tailed) were considered to be significant. All analyses were performed using the statistical software package SPSS (SPSS Japan Inc., Tokyo, Japan).

Results

Baseline Data

Among the 3,004 subjects, 127 (4.2%) had low ABI values, including four patients with symptoms of PAD. **Table 1** shows the baseline characteristics according to the ABI group. The patients with a low ABI were significantly older and had higher rates of diabetes, hypertension and a history of CHD, stroke and PAD, higher pulse pressure values and non-HDL cholesterol, triglyceride and uric acid levels and lower DBP, HDL and eGFR values. Data were available for 98.5% or more of the patients. The prevalence of a low ABI among the subjects with diabetes was 4.6% (120/2598), which was significantly higher than the 1.7% (7/406) observed in those without diabetes ($p=0.01$). Smoking was significantly associated with a low ABI following adjustment for age, sex and body mass index (BMI) (OR 1.87, 95%CI 1.27-2.77, $p<0.001$). A logistic regression analysis performed following adjustment for age, sex, BMI and smoking revealed that a low ABI was significantly associated with diabetes, a history of CHD, stroke and PAD, higher pulse pressure values and HbA_{1c}, non-HDL cholesterol, triglyceride and uric acid levels and lower diastolic pressure, HDL cholesterol and eGFR values.

Follow-Up

During a mean observation period of 4.4 years (range, 0.3-11.7), 93 deaths and 117 cardiovascular events (coronary heart disease: 39, stroke: 52, PAD: 5, sudden death: 21) occurred. A total of 866 subjects (28.8%) were lost to follow-up before reaching the end of study period in whom being free from events until the final visit was confirmed. The incidence of each outcome according to the ABI group is shown in **Table 2**. Compared with the subjects with an ABI of ≥ 0.9, those with an ABI of <0.9 had a significantly increased risk of an outcome event. When the analysis was confined to subjects with diabetes, a low ABI was

Table 1. Clinical characteristics of the subjects according to the ABI group at baseline

	ABI ≥ 0.9 $N=2877$	ABI < 0.9 $N=127$	OR (adjusted, 95%CI)
Age, years	59 ± 11	64 ± 15‡	-
Male, %	35	37	-
BMI, kg/m²	25.6 ± 4.1	26.1 ± 5.4	-
Smoking	33.5	38.6	-
Diabetes, %	86	94*	2.67 (1.23-5.86)*
Hypertension, %	55.6	69.3†	1.36 (0.91-2.03)
Antihypertensive agents, %	44.9	63.8‡	1.66 (1.12-2.45)*
Dyslipidemia, %	64.2	70.9	1.34 (0.90-1.99)
Lipid lowering agents, %	26.7	32.2	1.30 (0.88-1.91)
Systolic BP, mmHg	134 ± 21	136 ± 20	1.00 (0.99-1.01)
Diastolic BP, mmHg	76 ± 13	70 ± 13‡	0.97 (0.95-0.98)‡
Pulse pressure, mmHg	59 ± 15	66 ± 21‡	1.02 (1.01-1.03)†
HbA_{1c}, %	8.36 ± 2.09	8.44 ± 2.07	1.09 (1.01-1.19)*
Total cholesterol, mg/dL	205 ± 41	207 ± 39	1.00 (1.00-1.01)
HDL cholesterol, mg/dL	55 ± 14	50 ± 15†	0.97 (0.95-0.99)‡
Non-HDL cholesterol, mg/dL	150 ± 40	157 ± 37*	1.01 (1.00-1.01)†
Triglycerides, mg/dL§	134 (91-204)	156 (103-237)†	3.26 (1.57-6.76)†
eGFR, ml/min/1.73 m²	82.1 ± 22.4	71.6 ± 29.4	0.98 (0.97-0.99)†
Uric acid, μmol/L	300 ± 87	316 ± 87*	1.20 (1.05-1.37)†
History of CHD, %	2	7†	2.65 (1.26-5.57)*
History of stroke, %	4	19‡	4.03 (2.45-6.63)‡
History of PAD, %	0.1	11‡	142.6 (30.5-666.4)‡

The odds ratios (ORs) indicate the effects of the risk factors on the presence of a low ABI at baseline, as identified by a multiple logistic regression analysis following adjustment for age, sex, BMI and smoking. *$p<0.05$, †$p<0.001$, ‡$p<0.0001$. §Due to the skewed distribution, the median (interquartile range) is shown, and the OR indicates per log10 (triglycerides).

Table 2. Person-years (PY) at risk, number of cases and incidence (/1,000 person-years) of all-cause death, fatal and non-fatal cardiovascular events and the composite endpoint of death and cardiovascular events, according to the ABI group

Endpoint		*N*	PY at risk	No. of cases	Incidence (95% CI)	Adjusted HR (95% CI)
All-cause death	ABI ≥ 0.9	2877	12814	74	5.8 (4.5-7.2)	3.97 (2.29-6.88)
	ABI < 0.9	127	427	19	44.5 (27.0-68.6)	
Fatal and non-fatal cardiovascular event	ABI ≥ 0.9	2877	12859	101	7.9 (6.4-9.5)	2.79 (1.60-4.87)
	ABI < 0.9	127	396	16	40.4 (23.3-64.8)	
Cardiovascular event and all-cause death	ABI ≥ 0.9	2877	12859	156	12.1 (10.3-14.2)	2.86 (1.83-4.49)
	ABI < 0.9	127	396	25	63.1 (41.3-91.8)	

The HR was computed using a multivariate Cox regression analysis adjusted for age, sex, BMI and smoking.

found to exhibit independent associations with all three outcomes (adjusted HR [95% CI]; 4.15 (2.34-7.34); 2.50 (1.41-4.42); 2.85 (1.80-4.51), respectively for each outcome), which remained significant, even after adjustment for diabetes, hypertension, dyslipidemia, eGFR and a past history of CVD, in addition to age, sex, BMI and smoking. The times to event for all-cause death and the composite endpoint of cardiovascular events and all-cause death according to the ABI group are illustrated with Kaplan-Meier curves (**Fig. 1**).

The hazard ratios for the composite endpoint are shown in **Table 3**. The multivariate Cox regression analysis including all variables in **Table 3** revealed age, a low ABI, diabetes, a history of CVD and smoking to be independently and significantly predictive of the outcome. In order to explore the effects of a low ABI

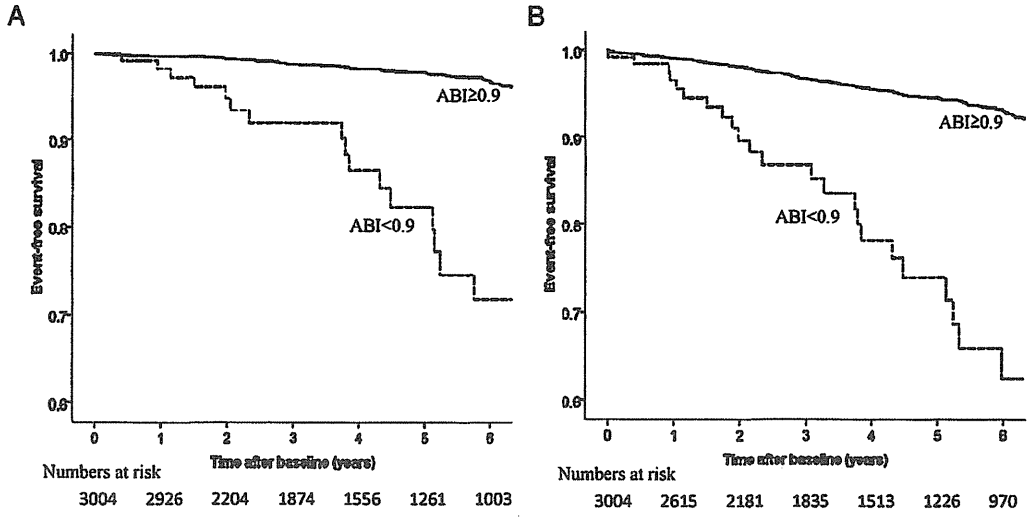

Fig. 1. Event-free survival according to the ABI group for all-cause death (A) and the composite endpoint of cardiovascular events and all-cause death (B). The Kaplan-Meier analysis shows significant differences between the two curves for the two outcomes ($p<0.0001$ according to the log-rank test, respectively).

on the presence or absence of each risk factor, the adjusted hazard ratios of a low ABI according to each risk group are shown in Table 3. The effects of a low ABI on the outcome were significant with respect to an age ≥ 70 years, diabetes, hypertension and no history of CVD or obesity. The effects of a low ABI remained significant irrespective of risk factors such as smoking, eGFR, gender and dyslipidemia.

Discussion

We found that the detection of a low ABI in routine clinical practice is highly predictive of all-cause death and cardiovascular events based on the results of our large-scale study of Japanese individuals, including subjects with and without diabetes. The predictive effect was significant and independent not only of conventional risk factors, but also a low eGFR and prevalent CVD, and remained significant in the subjects without a history of CVD. This study suggests that, even in a population characterized by a lower prevalence of obesity and PAD[13-15] and a lower incidence of CHD and PAD[13], the detection of a low ABI is useful for identifying diabetic subjects that should be targeted for multifactorial intensive treatment in the primary care setting in terms of improving all-cause mortality and cardiovascular morbidity.

The prevalence of PAD is higher in individuals with diabetes than in those without, as observed in this and other studies[5, 7, 10, 18], and is reportedly increasing[11]. The prevalence of diabetes and the number of elderly subjects with diabetes are also increasing in Japan, and 30-40% of people with diabetes smoke[13]. Only 3.1% (4/127) of such patients had symptoms of PAD among the subjects with a low ABI in this study, and underdiagnosis of PAD in primary care practice can be a barrier to effective secondary prevention of the high ischemic cardiovascular risks associated with PAD[4, 19]. Therefore, screening for low ABI values in routine clinical practice will become more important and possibly essential, particularly in individuals with diabetes and those receiving care for primary prevention of CVD.

The present study only incorporated the baseline measurements of ABI and other cardiovascular risk factors. Approximately 30-50% of the subjects had already received blood pressure- and/or lipid-lowering agents at baseline. This study did not investigate the effects of treatment, and further treatment was administered during the follow-up period (data not shown). It is presumed that a low ABI at baseline remains a risk factor for a poor outcome, even after the administration of aggressive treatment, which further reinforces the importance of routinely measuring the ABI.

We found a low ABI to be a risk factor, independent of other cardiovascular risk factors. Only a few studies have investigated the predictive value of a low ABI according to the presence or absence of CVD risk factors[3, 12]. In the present study, a low ABI was found to be an independent significant risk factor among

Table 3. Association of a low ABI and cardiovascular risk factors with subsequent development of the composite endpoint (all-cause death and cardiovascular events)

Risk factor		No.	PY* at risk	No. of cases	Incidence (95%CI)	HR of risk factor (adjusted, 95%CI)	HR of low ABI (adjusted, 95%CI)
Age (≥ 70 yr)	No	2473	10971	108	9.8 (8.1-11.9)	3.11 (2.20-4.41)	1.14 (0.45-2.88)
	Yes	531	2013	73	36.3 (28.5-45.4)		3.75 (2.04-6.90)
ABI<0.9	No	2877	12589	156	12.1 (10.3-14.2)	2.52 (1.54-4.12)	-
	Yes	127	396	25	63.1 (41.3-91.8)		-
Diabetes	No	406	1483	8	5.4 (2.3-10.6)	2.41 (1.18-4.94)	2.29 (0.10-50.68)
	Yes	2598	11501	173	15.0 (12.9-17.4)		2.22 (1.32-3.73)
History of CVD	No	2822	12262	193	15.7 (13.6-18.1)	1.87 (1.24-2.82)	2.99 (1.55-5.79)
	Yes	181	723	39	53.9 (38.6-73.0)		1.77 (0.83-3.77)
Smoking (current)	No	1961	8448	116	13.7 (11.4-16.4)	1.42 (1.02-1.97)	2.16 (1.16-4.01)
	Yes	998	4506	65	14.4 (11.1-18.3)		2.40 (1.03-5.59)
eGFR	No	2555	11145	128	11.5 (9.6-13.6)	1.43 (0.99-2.07)	2.51 (1.21-5.19)
(<60 ml/min/1.73 m^2)	Yes	425	1799	53	29.5 (22.1-38.4)		2.53 (1.30-4.93)
Hypertension	No	1314	5074	60	11.8 (9.0-15.2)	1.35 (0.97-1.88)	3.06 (0.99-9.51)
	Yes	1687	7203	121	16.8 (14.0-20.0)		2.49 (1.44-4.32)
BMI (≥ 30 kg/m^2)	No	2596	11299	158	14.0 (11.9-16.3)	1.06 (0.67-1.67)	3.21 (1.90-5.42)
	Yes	408	1685	23	13.6 (8.7-20.4)		0.94 (0.20-4.36)
Male sex	No	1957	8771	132	15.1 (12.6-17.8)	0.83 (0.59-1.18)	2.53 (1.44-4.45)
	Yes	1047	4214	49	11.6 (8.6-15.4)		2.92 (1.04-8.23)
Dyslipidemia	No	1068	4726	80	16.9 (13.4-21.0)	0.72 (0.53-1.01)	4.14 (1.89-9.10)
	Yes	1936	8258	101	12.2 (10.0-14.8)		1.98 (1.05-3.72)

The hazard ratio of each risk factor is indicated as the result of a Cox regression analysis adjusted for all variables in the Table. The hazard ratio of a low ABI according to the presence or absence of each risk factor is simultaneously indicated as the result of a Cox regression analysis adjusted for all variables in the Table.
*PY: person-years

subjects with an age of ≥ 70, hypertension, a BMI of <25 and no history of CVD, which is in agreement with the findings of some, but not all previous studies[3, 12], whereas most other studies did not specifically examine this issue. While the present subjects with a low ABI more often exhibited a history of CVD, we found that a low ABI was predictive of CVD among the subjects without prevalent CVD.

It was interesting to find an association between hyperuricemia and a low ABI in the baseline analysis. This is the first report of such an association to our knowledge, and the results are in line with the findings of several studies showing a relationship between the serum uric acid level and the development of atherosclerotic disease[20, 21]. A significant association between a reduced eGFR and a low ABI has previously been reported[22], and this finding was confirmed in our study at baseline.

Several limitations of the present study should be mentioned. First, we should acknowledge the small number of events in the non-diabetic subjects, who demonstrated a lower prevalence of a low ABI. Second, the generalizability of the subjects should be discussed. The incidence of all-cause death and cardiovascular events observed in the subjects with and without diabetes in this cohort was slightly lower and/or almost the same as that observed in Japanese populations reported in other studies[23-26]. These facts support the generalizability of the cohort. Third, approximately 29% of the participants were lost to follow-up because they moved to other cities/clinics or discontinued clinic attendance. We were unable to evaluate their further outcomes because no regional or national registries for death and disease identification systems are available in Japan. In order to minimize this inherent problem, a life-table analysis was used to cover the

censored cases. Finally, whether an ABI of >1.4 occurs more commonly in subjects with diabetes and is associated with mortality requires further investigation.

In conclusion, the present study suggests that a low ABI is an integrated marker of tissue/vascular damage affected by age, smoking, blood pressure, blood glucose, lipids, uric acid, the renal function and prevalent CVD, indicating its role as an excess and independent risk factor for all-cause death and cardiovascular events.

Disclosures

None.

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Clin Exp Nephrol (2014) 18:584–592
DOI 10.1007/s10157-013-0922-5

REVIEW ARTICLE

Macrophage-mediated glucolipotoxicity via myeloid-related protein 8/toll-like receptor 4 signaling in diabetic nephropathy

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Received: 16 April 2013 / Accepted: 28 November 2013 / Published online: 20 December 2013

Abstract Dyslipidemia is an independent risk factor for the development and progression of diabetic nephropathy (DN). In this review, we summarize mouse models with both diabetes and dyslipidemia, and their associated complications. We then discuss molecules potentially involved in deterioration of DN by dyslipidemia. We focus especially upon toll-like receptor 4 (TLR4) and one of its endogenous ligands, myeloid-related protein 8 (MRP8 or S100A8), since we have found that their mRNA levels are commonly increased in glomeruli of type 1 (streptozotocin [STZ]-induced) and type 2 (*A-ZIP/F-1* lipoatrophic) diabetic mice. Gene expression of *MRP8* and *Tlr4* is further upregulated during worsening of STZ-induced DN by a high fat diet (HFD). Moreover, these HFD-induced changes are accompanied by enhanced gene expression of *CCAAT element binding protein β* and phosphorylation of c-Jun N-terminal kinase in the kidney, which have also been reported in pancreatic β cells under diabetic-hyperlipidemic conditions. Effects of a HFD upon DN are cancelled in *Tlr4* knockout mice. Macrophages are the predominant source of MRP8 in glomeruli. In cultured macrophages, combinatorial treatment with high glucose and palmitate amplifies *MRP8* expression in a *Tlr4*-dependent manner, and recombinant MRP8 protein markedly increases gene expression of the inflammatory cytokines *interleukin-1β* and *tumor necrosis factor α*. Here, we propose 'macrophage-mediated glucolipotoxicity' via activation of MRP8/TLR4 signaling as a novel mechanism of pathophysiology for DN.

Keywords Diabetic nephropathy · Glucolipotoxicity · Macrophage · Toll-like receptor

Introduction

Since only one-third of patients with type 1 diabetes develop diabetic nephropathy (DN), we should consider the role of factors other than hyperglycemia in the pathophysiology of DN, including genetic, epigenetic, environmental and metabolic aspects. Several reports describe hyperlipidemia or dyslipidemia as an independent risk factor for the progression of DN in type 1 and type 2 diabetes, as well as for atherosclerotic complications [1–4]. Using type 1 (streptozotocin [STZ]-induced) and type 2 (*db/db*) diabetic mouse models, we have confirmed that treatment of diabetic mice with a high fat diet (HFD) exacerbates albuminuria and glomerular lesions [5]. Of note, single nucleotide polymorphisms in *acetyl-CoA carboxylase β* gene, which plays an important role in the regulation of fatty acid metabolism, exhibit a potent association with proteinuria in patients with type 2 diabetes [6, 7]. Accordingly, a concept of synergistic toxicity caused

This article was, in part, presented at the 43rd Western Regional Meeting of the Japanese Society of Nephrology, held at Matsuyama, Japan, in 2013.

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by glucose and lipid, described as 'glucolipotoxicity', has emerged in recent years. However, the underlying molecular mechanism is still obscure, especially in renal complication [8]. Here we will discuss diabetic-hyperlipidemic mouse models and glucolipotoxicity in the kidney.

Diabetic-hyperlipidemic mouse models

As described above, several clinical and experimental phenomena have highlighted the synergistic effects of hyperglycemia and hyperlipidemia upon the development and progression of diabetic complications including nephropathy. Despite the fact that there are several limitations associated with the difference in hyperlipidemia between rodents and humans, mouse models are still most widely used to study complications caused by diabetes and hyperlipidemia. The reasons include small animal size, short generation time, the ease of induction of diabetes, hyperlipidemia or gene manipulation, and cost effectiveness [9]. Hence, in the last decade diabetic-hyperlipidemic mouse models have been used for genetic modification, pharmacological treatment and/or some particular chow diets that abundantly contain fat and/or cholesterol. In this section, representative mouse models are summarized.

Apolipoprotein E-deficient mice treated with streptozotocin (*ApoE* KO + STZ)

ApoE KO + STZ mice are one of the most popular diabetic-hyperlipidemic mouse models. This model shows not only hypercholesterolemia and hypertriglyceridemia, but also accelerated aortic atherosclerotic lesions [10–12] and nephropathy [13–15] associated with diabetes. These reports revealed that advanced glycation end-products [13, 14] and endoplasmic reticulum (ER) stress [16, 17] are candidate mediators of glucolipotoxicity in *ApoE* KO + STZ mice.

Low-density lipoprotein (LDL) receptor-deficient mice treated with STZ (*LDLR* KO + STZ)

LDLR KO + STZ mice show dyslipidemia including high LDL cholesterol, low high-density lipoprotein (HDL) cholesterol levels and hypertriglyceridemia, mimicking human metabolic syndrome [18]. Moreover, addition of a HFD exacerbates hypertriglyceridemia, hypercholesterolemia, and diabetic renal lesions (including glomerular and tubulointerstitial macrophage infiltration) in this model [19]. The authors [19] referred to an earlier work indicating that irradiation-induced depletion of bone marrow cells (including monocytes) reduces renal injury in STZ-diabetic rats [20].

STZ-induced diabetic mice with HFD feeding (STZ + HFD)

A supplemental HFD on STZ-treated diabetic mice increases blood triglyceride and free fatty acid concentrations, at least in part, because of insulin deficiency, suggesting that this model might be useful especially for analyzing pathophysiology by high triglyceride-rich lipoprotein and/or high free fatty acids coexisting with high glucose conditions. In STZ + HFD mice, there are several reports describing vascular complications such as cardiovascular dysfunction [21], retinopathy [22], neuropathy [23] and nephropathy [5, 24].

Treatment of wild-type mice with STZ and HFD synergistically increases albuminuria [5] and expands mesangial area (Fig. 1). Induction of diabetes by STZ causes a marked increase in urine volume and creatinine clearance of normal diet-fed and HFD-fed animals, respectively, suggesting that glomerular hyperfiltration has occurred. On the other hand, HFD treatment reduces urine volume and creatinine clearance in STZ mice (Fig. 1), suggesting that HFD is not causing more hyperfiltration but is causing non-hemodynamic actions which will be discussed below.

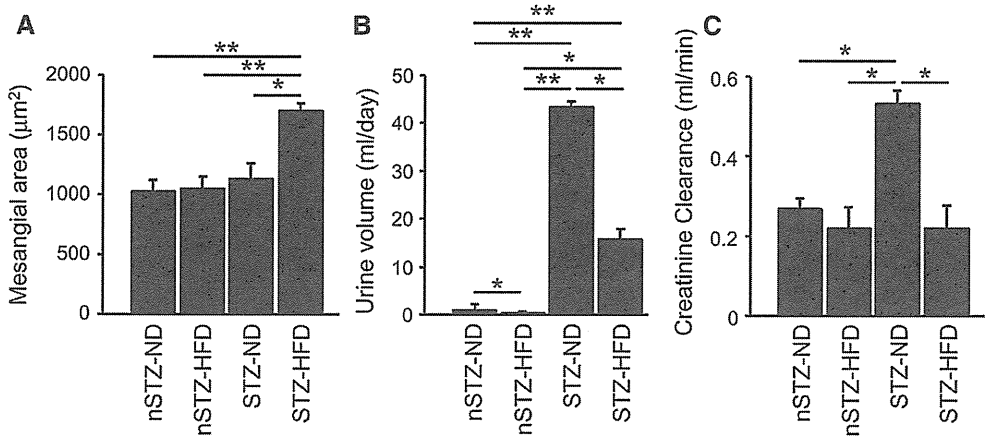

Fig. 1 Effects of STZ and/or HFD upon mesangial expansion (**a**), urine volume (**b**) and creatinine clearance (**c**) in wild-type mice. *nSTZ-ND* non STZ-normal diet, *nSTZ-HFD* non STZ-high fat diet, *STZ-ND* STZ-normal diet, *STZ-HFD* STZ-high fat diet. Data are mean ± SEM. $n = 4$–11. $^{*}p < 0.01$, $^{**}p < 0.001$. Modified from Kuwabara and others [5]

A-ZIP/F-1 lipoatrophic diabetic mice

A-ZIP/F-1 mice are a genetic mouse model of lipoatrophic diabetes, characterized by severe insulin resistance, dyslipidemia including hypertriglyceridemia and high free fatty acids, and fatty liver [25, 26]. This model is based upon dominant-negative expression of B-ZIP transcription factors of both C/EBP and Jun families under the control of aP2 enhancer/promoter, causing paucity of adipose tissue. *A-ZIP/F-1* mice may serve as a useful tool for studying DN, because they manifest severe nephrotic syndrome and typical histopathological renal lesions which are glomerular hypertrophy, diffuse and pronounced mesangial expansion and accumulation of extracellular matrix [27]. Notably, these renal changes are reversible to some extent by replacement therapy with a fat-derived hormone leptin [27].

Other mouse models

There are a few other diabetic-hyperlipidemic mouse models such as non-obese diabetic mice or *Ins2*^{Akita} diabetic mice combined with HFD feeding [28, 29], but their renal involvement has not been characterized well. Regardless of the models described above, differences in genetic backgrounds critically affect glucose and lipid metabolism among mouse strains [30]. Furthermore, even similar levels of hyperglycemia cause distinct renal changes among different strains and species. For instance, the DBA/2 strain is highly susceptible to DN, whereas the C57BL/6 strain is relatively resistant [31–33]. In addition, since cholesteryl ester transfer protein is inactive in rodents, HDL is the dominant lipoprotein in mice [34]. Apolipoprotein B in rodents also differs from that in humans [35].

Molecules involved in glucolipotoxicity in the kidney and pancreatic β cells

Although glucotoxicity and lipotoxicity were originally proposed as independent concepts, Prentki et al. reported a novel concept of glucolipotoxicity in pancreatic β cells in 1996. They reported that elevated ambient levels of glucose and free fatty acid cause synergistic inhibition of insulin secretion [36]. On the other hand, they reported that increased intracellular glucose-derived metabolites inhibit enzymes for β-oxidation, leading to cytosolic accumulation of lipids [37]. Subsequently, there have been several reports about the molecular mechanism underlying glucolipotoxicity involved in pancreatic β cell dysfunction and insulin resistance [38–40]. Furthermore, phenomena of glucolipotoxicity are also observed in DN of humans [1–4] and rodents [41, 42], but their pathophysiology remains largely unknown [8]. Here, we will compare glucolipotoxicity upon pancreatic β cell dysfunction and DN.

c-Jun N-terminal kinase (JNK)

JNK plays a pivotal role in ER stress-induced 'unfolded protein response' in innate immune system [43]. It was later revealed that ER stress-induced JNK activation is associated with chronic inflammation or high ambient fatty acid levels in obesity or type 2 diabetes [44, 45]. In pancreatic β-cells, high glucose concentrations augment lipotoxicity through JNK activation, at least partly, in an ER stress-dependent manner [46, 47]. In our diabetic-hyperlipidemic model [5], treatment with STZ and HFD synergistically increases phosphorylation of IκB and mRNA expression of pro-inflammatory genes in the kidney, in parallel with phosphorylation of JNK, but not with phosphorylation of other mitogen-activated protein (MAP) kinases such as p38 or extracellular signal-regulated kinase (ERK) (Fig. 2).

CCAAT element binding protein beta (C/EBPβ)

CCAAT element binding protein beta (C/EBPβ) is one of the transcriptional repressors of insulin gene and induced

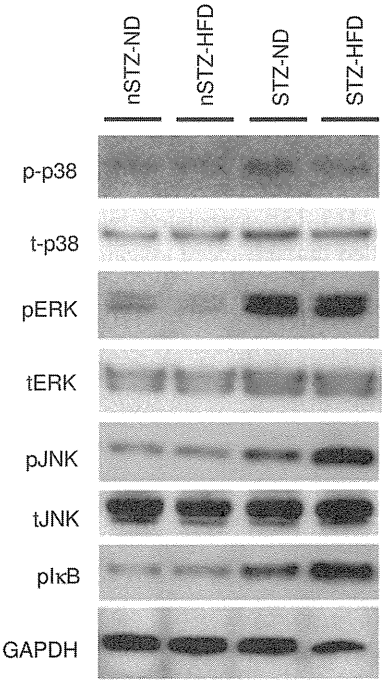

Fig. 2 Western blot analysis for phosphorylation of MAP kinases and IκB in kidney of STZ + HFD mice. *p-/t-p38* phosphorylated/total p38 MAP kinase, *p/tERK* phosphorylated/total extracellular signal-regulated kinase, *p/tJNK* phosphorylated/total c-Jun N-terminal kinase, *pIκB* phosphorylated inhibitor of κB. Modified from Kuwabara and others [5]

by chronic hyperglycemia [48]. C/EBPβ is increased by fatty acids through the Per-Arnt-Sim kinase (PASK) pathway [49] in pancreatic β cells. Since PASK is also induced by high glucose conditions, these mechanisms may possibly exert glucolipotoxic effects. In the kidney, C/EBPβ is increased in diabetic rats, but not other C/EBP isoforms [50]. Furthermore, renal upregulation of *C/EBPβ* mRNA in STZ-induced diabetic mice is further enhanced by additional HFD feeding in our experiments [5].

Of note, JNK/AP-1 and C/EBPβ pathways may also contribute to glucolipotoxicity-induced renal damage through upregulation of myeloid-related protein 8 (MRP8, also known as S100A8 or calgranulin A), whose gene promoter region contains AP-1 binding site [51, 52] and C/EBP motif [53, 54], as discussed in the next section.

Fetuin A

Over the last few years, there has been growing evidence for fatty acid-induced lipotoxicity, such as insulin resistance, through toll-like receptor 4 (TLR4) [55–57]. However, it is still controversial whether fatty acid stimulates TLR4 directly or indirectly. Recently, fetuin A has been identified as an adopter protein combining fatty acids and TLR4 [58], and its plasma levels are elevated in diabetic humans and mice [59, 60]. ER stress induced by high glucose and palmitate increases the expression of fetuin A [60], suggesting that fetuin A could hypothetically participate in glucolipotoxicity upon macrophages.

MRP8/TLR4

MRP8 was originally identified as a cytoplasmic calcium-binding protein in neutrophils and monocytes [61]. MRP8, by making a heterodimer with MRP14 (or S100A9), has become widely recognized as a potent endogenous ligand for TLR4 in various diseases including septic shock and vascular and autoimmune disorders [62–64]. To identify candidate disease-modifying molecules in DN, we have performed microarray analysis using isolated glomeruli from two different diabetic models of mice—STZ-induced insulin-dependent diabetic mice and lipoatrophic insulin-resistant A-ZIP/F-1 mice. We then focused upon *MRP8* and *Tlr4*, because expression of both genes is commonly increased in these two models [5]. It is noteworthy that diabetic-hyperlipidemic mice such as STZ-HFD mice or *A-ZIP/F-1* mice show remarkable upregulation of *MRP8* and *Tlr4* compared to control non-diabetic mice (Fig. 3). Since macrophages are identified as the major source of MRP8 in the glomeruli of STZ-HFD mice [5], we examined the effects of high glucose and fatty acid on the expression of *MRP8* (Fig. 4) and *Tlr4* in cultured macrophages. This in vitro study showed that treatment with fatty acid

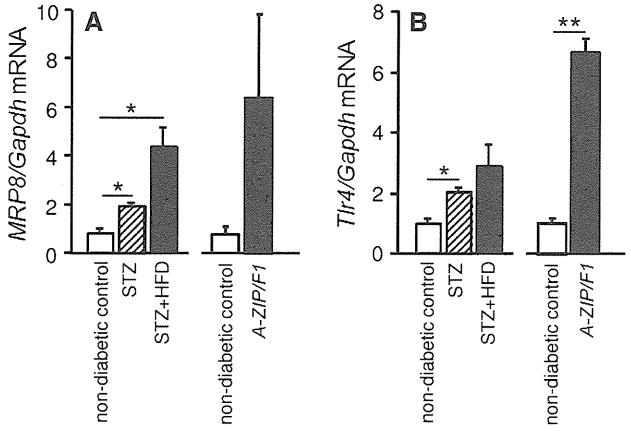

Fig. 3 Glomerular gene expression of *MRP8* (**a**) and *Tlr4* (**b**) in STZ + HFD and lipoatrophic *A-ZIP/F-1* mice determined by TaqMan real-time PCR. *White bars* non-diabetic control group, *striped bars* diabetic group, *black bars* diabetic-hyperlipidemic group. Data are mean ± SEM. $n = 4$–7. $*p < 0.01$, $**p < 0.001$. Modified from Kuwabara and others [5]

amplifies *MRP8* expression only under high ambient glucose conditions. Although *Tlr4* is expressed slightly more in high glucose conditions than in low glucose conditions, fatty acid does not alter *Tlr4* expression [5]. In addition, synergistic effects with high glucose and fatty acid on macrophages and diabetic kidneys are abrogated by *Tlr4* deletion [5] (Fig. 4). Moreover, we have observed that recombinant MRP8 protein markedly increases gene expression of the inflammatory cytokines *interleukin-1β* and *tumor necrosis factor α* (*TNF-α*) in cultured macrophages (submitted) [62]. Similarly, macrophages also play an important role in insulin resistance and β-cell dysfunction through fatty acid-induced TLR4 activation [65, 66]. Particularly in the kidney, MRP8 produced by infiltrated macrophages might exert glucolipotoxic effects upon diabetic glomeruli in a paracrine manner, potentially leading to mesangial expansion, podocyte injury, glomerular sclerosis and albuminuria (Fig. 5), because TLR4 is reportedly expressed in healthy or injured glomerular intrinsic cells including mesangial cells [67, 68], endothelial cells [67, 69] and podocytes [70, 71]. Taken together, we propose 'macrophage-mediated glucolipotoxicity' via activation of MRP8/TLR4 signaling as a novel concept for pathophysiology of DN (Fig. 5).

To understand the clinical implication of MRP8 expression in humans, we have carried out immunohistochemical analysis of MRP8 expression in renal biopsy samples from patients with DN, obesity-related glomerulopathy (ORG) and non-obese, non-diabetic controls (which are minor glomerular abnormality [MGA] and minimal change nephrotic syndrome [MCNS]). We have not been able to obtain reliable antibody against TLR4 to date. The rank orders of glomerular and tubulointerstitial MRP8 protein expression levels

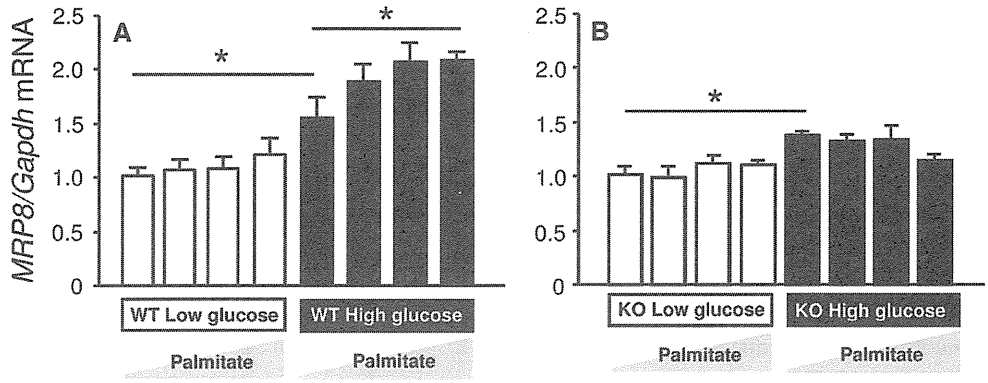

Fig. 4 Gene expression of *MRP8* and effects of glucose or fatty acid in bone marrow-derived macrophages (BMDMs) determined by TaqMan real-time PCR. BMDMs generated from wild-type (WT, **a**) or *Tlr4* knockout (KO, **b**) mice were cultured under low-glucose (100 mg/dl, *white bars*) or high-glucose (450 mg/dl, *black bars*) conditions, and were stimulated with palmitate (0, 10, 50, and 200 μM, respectively, from the *left*) for 24 h. Data are mean ± SEM. $n = 6$. $^*p < 0.05$. Modified from Kuwabara and others [5]

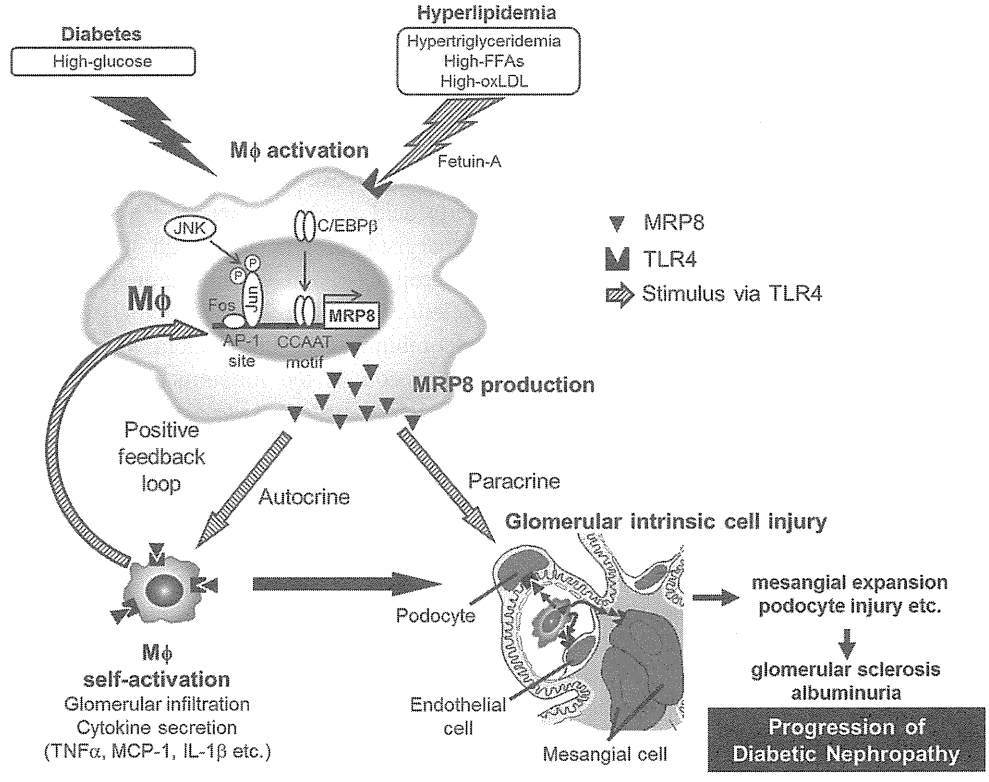

Fig. 5 Proposed mechanism of macrophage-mediated glucolipotoxicity in diabetic nephropathy. Hyperlipidemia (or high free fatty acids) activates circulating macrophages through TLR4-mediated upregulation of MRP8, specifically under hyperglycemic conditions. These synergistic effects upon MRPã8 production in macrophages might be mediated by fetuin A and transcription factors AP-1 and CEBP/β. Macrophage activation is enhanced by a positive feedback, mediated by MRP8/TLR4 interaction in an autocrine fashion. Since glomerular intrinsic cells (such as podocytes, mesangial cells and endothelial cells) reportedly express TLR4, they can be activated through multiple pathways including (1) MRP8 from blood circulation, (2) MRP8 and inflammatory cytokines produced by glomerulus-infiltrating macrophages, and (3) hyperlipidemia. Activation of glomerular cells results in mesangial expansion and podocyte injury, further leading to glomerular sclerosis (fibrosis) and albuminuria

are DN > ORG > MCNS > MGA. Glomerular MRP8 expression is strongly correlated to the extent of proteinuria at 1 year after renal biopsy, whereas tubulointerstitial MRP8 expression is associated with worsening of renal function within a year, suggesting that renal MRP8 expression may become a new biomarker for DN (submitted).

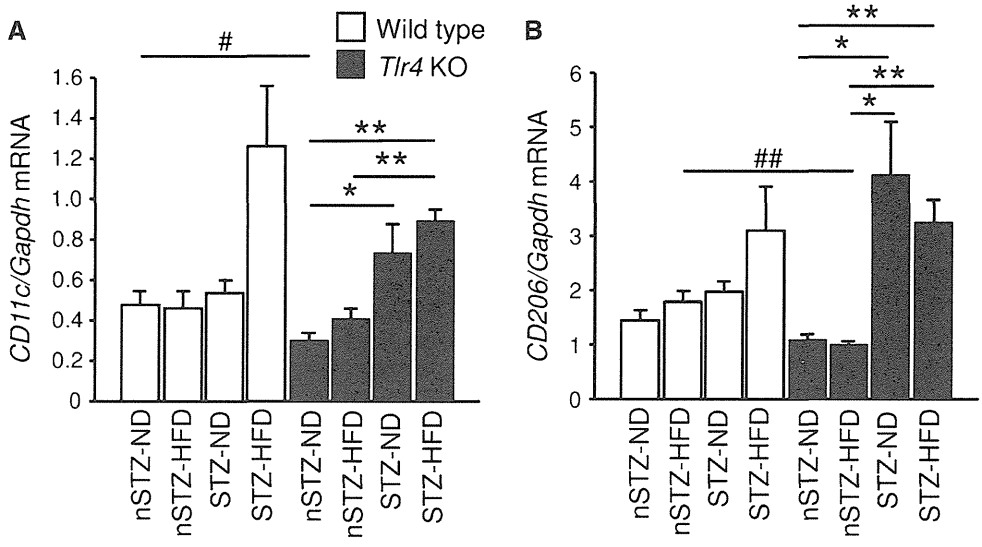

Fig. 6 Glomerular gene expression of M1 (**a**) and M2 (**b**) macrophage markers in STZ-HFD mice determined by TaqMan real-time PCR. Data are mean ± SEM. $n = 4$–11. $^{*}p < 0.05$, $^{**}p < 0.01$. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ for similarly treated *Tlr4* KO versus wild-type

The role of M1 and M2 macrophages in DN with glucolipotoxicity

There are several subtypes of macrophages including M1 and M2 in tissue injury and repair [72–74]. During the course of renal ischemia/reperfusion injury [75] and unilateral ureteral obstruction [76], switch from proinflammatory M1 to anti-inflammatory or profibrotic M2 subtype occurs in macrophages infiltrating the tubulointerstitium. Here, we have carried out preliminary analysis of M1 and M2 macrophages in glomeruli of STZ + HFD mice by studying gene expression levels of *CD11c* (or *Itgax*) and *CD206* (or *Mrc1*) as markers of M1 and M2 subtypes, respectively [77, 78] (Fig. 6). In wild-type mice, treatment with STZ alone does not affect glomerular expression of *CD11c* and *CD206* genes, and addition of HFD to STZ causes a 100 % increase in *CD11c* and a 30 % increase in *CD206*, suggesting relative predominance of M1 subtype in diabetic-hyperlipidemic conditions. Furthermore, in *Tlr4* KO mice, the stimulatory effects of HFD upon STZ treatment are canceled both for *CD11c* and *CD206* genes, and simple STZ treatment increases *CD11c* expression by two-fold and increases *CD206* expression by three-fold, suggesting the presence of M2 predominant status. These results imply that TLR4-mediated signal is partially suppressing M2 subtype in STZ-normal diet mice and enhancing M1 subtype in STZ-HFD mice. These findings are in good agreement with previous reports indicating that treatment of macrophages with MRP8 induces M1 subtype (through TLR4 as lipopolysaccharide does) [61, 72, 76] and MRP8-expressing macrophages exhibits M1 characteristics by secretion of TNF-α and interleukin-6 [74, 79]. Formally, M1/M2 subtype analysis had to be carried out by analyzing isolated macrophages extracted from tissues.

Furthermore, in STZ + HFD animals, the levels of macrophage infiltration and extracellular matrix accumulation are proportional and progressive, suggesting that M1–M2 switching does not occur spontaneously in this model of DN. In glomeruli of STZ + HFD mice, >80 % of MRP8 signals co-localize with macrophage marker Mac2 (or Lgals3) [5], whereas collecting duct epithelial cells are the main source of MRP8 expression in unilateral ureteral obstruction [76].

In conclusion, a number of epidemiological and experimental studies have revealed that glucotoxicity and lipotoxicity cause synergistic effects upon the development and progression of DN. Macrophages have emerged as a potential contributor for mediating glucolipotoxicity through activation of MRP8/TLR4 signaling in diabetic glomeruli in our experiments. Although further studies are needed to understand regulation and potential role of MRP8/TLR4 signaling, targeting key molecules involved in this pathway may lead to novel therapeutic strategy to combat DN.

Acknowledgments This work was supported in part by Grant-in-Aid for Diabetic Nephropathy and Nephrosclerosis Research from the Ministry of Health, Labour and Welfare of Japan (KM), research grants from the Japanese Ministry of Education, Culture, Sports, Science and Technology (TK, KM and MM), from the Japan Foundation for Applied Enzymology (TK), from the Smoking Research Foundation (MM) and from the ONO Medical Research Foundation (TK).

Conflict of interest The authors have declared no competing interest.

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