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REVIEW ARTICLE

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

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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

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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

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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.

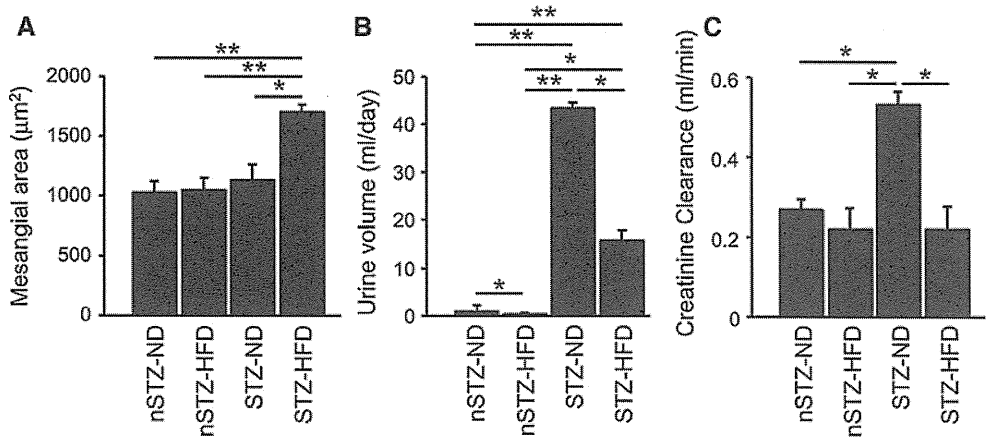

Fig. 1 Effects of STZ and/or HFD upon mesangial expansion (**a**), urine volume (**b**) and creatinine clearance (**c**) in wild-type mice. *nSTZ-ND* non STZ-normal diet, *nSTZ-HFD* non STZ-high fat diet, *STZ-ND* STZ-normal diet, *STZ-HFD* STZ-high fat diet. Data are mean ± SEM. $n = 4$–11. $^{*}p 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

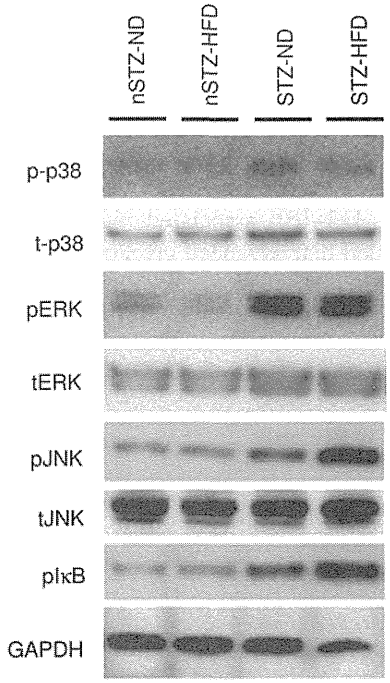

Fig. 2 Western blot analysis for phosphorylation of MAP kinases and IκB in kidney of STZ + HFD mice. *p-/t-p38* phosphorylated/total p38 MAP kinase, *p/tERK* phosphorylated/total extracellular signal-regulated kinase, *p/tJNK* phosphorylated/total c-Jun N-terminal kinase, *pIκB* phosphorylated inhibitor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

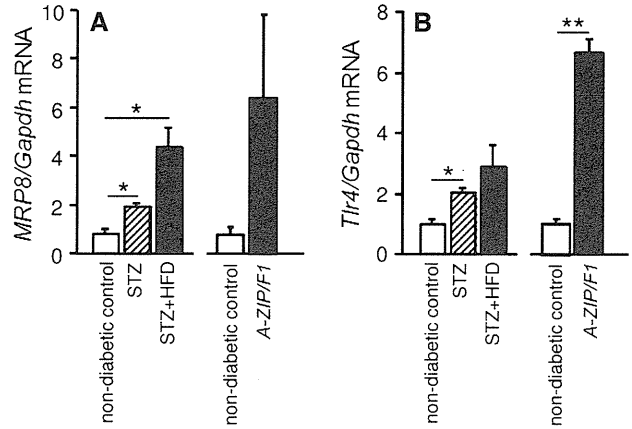

Fig. 3 Glomerular gene expression of *MRP8* (**a**) and *Tlr4* (**b**) in STZ + HFD and lipoatrophic *A-ZIP/F-1* mice determined by TaqMan real-time PCR. *White bars* non-diabetic control group, *striped bars* diabetic group, *black bars* diabetic-hyperlipidemic group. Data are mean ± SEM. $n = 4$–7.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

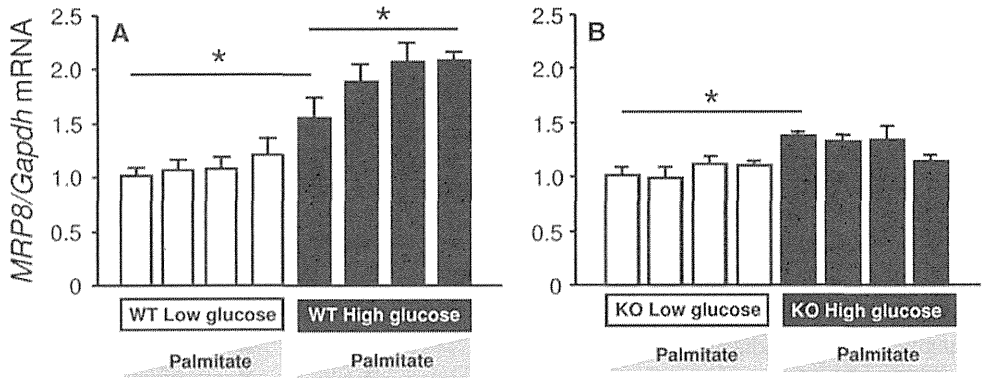

Fig. 4 Gene expression of *MRP8* and effects of glucose or fatty acid in bone marrow-derived macrophages (BMDMs) determined by TaqMan real-time PCR. BMDMs generated from wild-type (WT, **a**) or *Tlr4* knockout (KO, **b**) mice were cultured under low-glucose (100 mg/dl, *white bars*) or high-glucose (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]

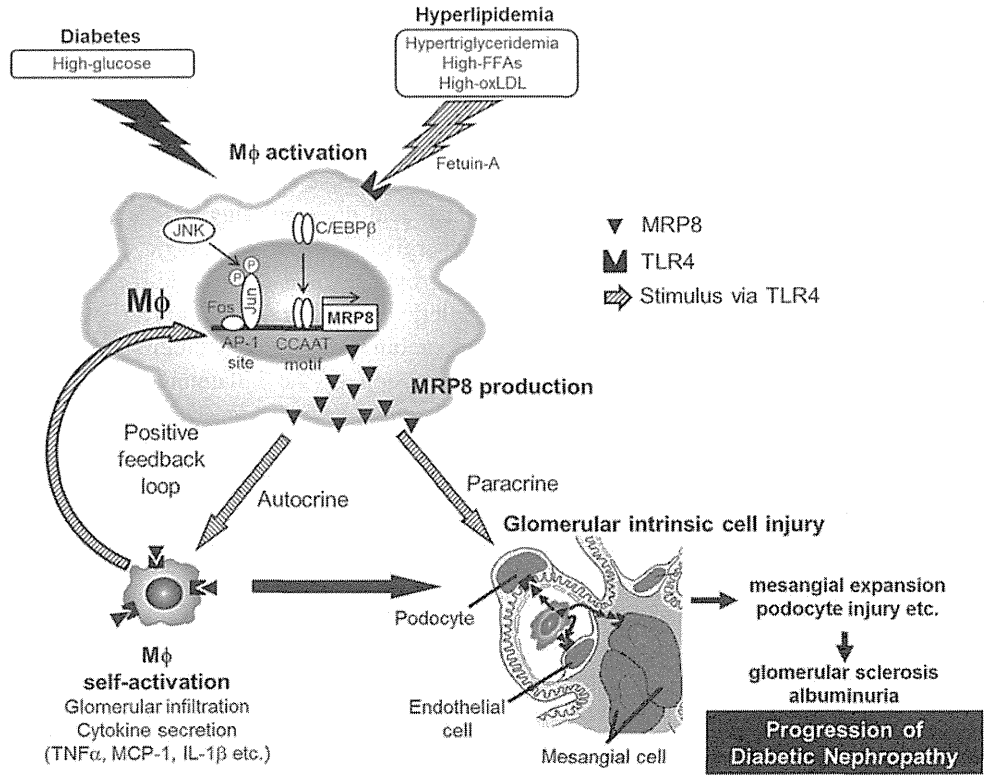

Fig. 5 Proposed mechanism of macrophage-mediated glucolipotoxicity in diabetic nephropathy. Hyperlipidemia (or high free fatty acids) activates circulating macrophages through TLR4-mediated upregulation of MRP8, specifically under hyperglycemic conditions. These synergistic effects upon MRPā8 production 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).

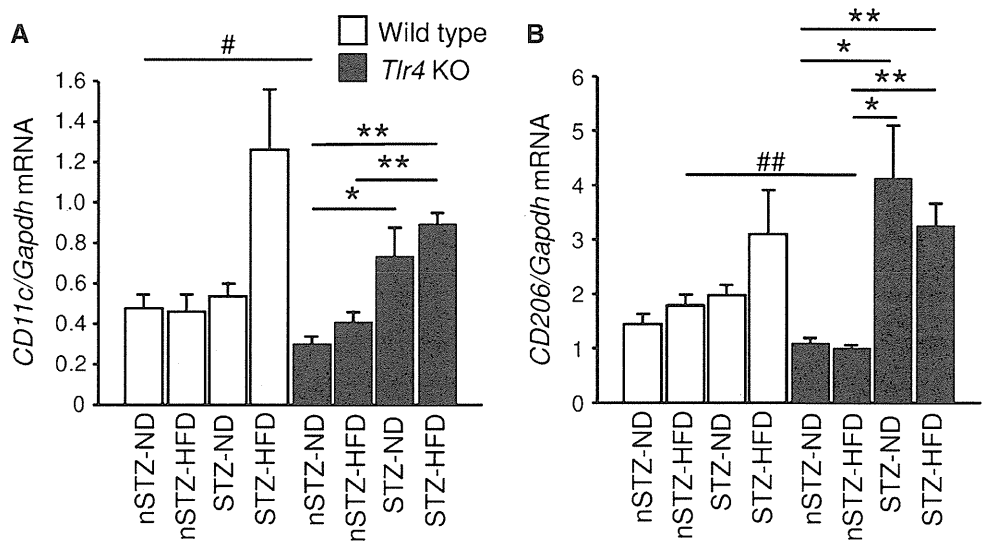

Fig. 6 Glomerular gene expression of M1 (**a**) and M2 (**b**) macrophage markers in STZ-HFD mice determined by TaqMan real-time PCR. Data are mean ± SEM. $n = 4$–11. $^{*}p < 0.05$, $^{**}p < 0.01$. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ for similarly treated *Tlr4* KO versus wild-type

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.

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Conflict of interest The authors have declared no competing interest.

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SPECIAL ARTICLE

A new classification of Diabetic Nephropathy 2014: a report from Joint Committee on Diabetic Nephropathy

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Abstract The Joint Committee on Diabetic Nephropathy has revised its Classification of Diabetic Nephropathy (Classification of Diabetic Nephropathy 2014) in line with the widespread use of key concepts such as the estimated glomerular filtration rate (eGFR) and chronic kidney disease. In revising the Classification, the Committee carefully evaluated, as relevant to current revision, the report of a study conducted by the Research Group of Diabetic Nephropathy, Ministry of Health, Labour and Welfare of Japan. Major revisions to the Classification are summarized as follows: (1) eGFR is substituted for GFR in the Classification; (2) the subdivisions A and B in stage 3 (overt nephropathy) have been reintegrated; (3) stage 4 (kidney failure) has been redefined as a GFR less than 30 mL/min/1.73 m^2, regardless of the extent of albuminuria; and (4) stress has been placed on the differential diagnosis of diabetic nephropathy versus non-diabetic kidney disease as being crucial in all stages of diabetic nephropathy.

Keywords Diabetic nephropathy · Chronic kidney disease (CKD) · Albuminuria · Proteinuria · Glomerular filtration rate (GFR)

Japan Diabetes Society, Japanese Society of Nephrology, Japanese Society for Dialysis Therapy, and Japan Society of Metabolism and Clinical Nutrition established the Joint Committee on Diabetic Nephropathy, which published the revised Classification of Diabetic Nephropathy 2014 in Japanese [1–4]. This is the English version of that revision.

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Introduction

Diabetic nephropathy became the leading cause of chronic dialysis in 1998. Since then, the incidence of this condition has increased with only a recent plateau. However, diabetic nephropathy continues to account for a large proportion of all cases of chronic kidney disease (CKD) and remains by far the most common underlying cause of chronic dialysis among all kidney diseases [5], consequently leading to the escalation of healthcare costs, thus representing a compelling medico-social issue of interest.

The Classification of Diabetic Nephropathy (hereafter "Classification") developed earlier by the Research Group

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of Diabetic Nephropathy at the Ministry of Health, Labour and Welfare (MHLW) [6] and later revised by the Joint Committee on Diabetic Nephropathy (hereafter "Committee") [7] is widely used in Japan. However, as the concept of CKD was proposed, followed by the classification of CKD stages [8], it became clear that there exists a subpopulation of patients with discrepant classifications of diabetic nephropathy and CKD. This is thought to be due to the fact that diabetic nephropathy is primarily classified according to the extent of albuminuria in addition to the glomerular filtration rate (GFR) (i.e., creatinine clearance [CCr]), whereas CKD is primarily classified based on the estimated GFR [estimated GFR (eGFR)]. Meanwhile, eGFR has become increasingly used to assess GFR, and a new classification of CKD was developed in 2012 [9]. Against this background, the Committee therefore discussed issues of interest in depth and sought to develop a revision of the Classification.

Development of the 2014 Classification (Revised Classification) (see Table 1)

Prior to revising the Classification, as part of a MHLW-subsidized project on kidney disease, entitled "Diabetic Nephropathy Research, from the Ministry of Health, Labour and Welfare of Japan", a "historical cohort study" was conducted by the Research Group of Diabetic Nephropathy, MHLW, involving a total of 4,355 subjects with type 2 diabetes from 10 participating healthcare facilities with the aim of evaluating renal events (i.e., a decrease in eGFR to half the baseline level and/or the need for dialysis), cardiovascular events and all-cause mortality [10, 11]. Summarized below are the major findings of this study (for detailed information, please access the MHLW website http://www.mhlw.go.jp/ or refer to the literature cited above).

1. Renal and cardiovascular events and all-cause mortality were significantly increased in the subjects with micro- or macroalbuminuria compared to that observed in the subjects with normoalbuminuria.
2. In those with renal impairment (defined as a GFR less than 60 mL/min/1.73 m^2):
   a. The risk of renal events increased in association with the onset of microalbuminuria and further increased with the onset of macroalbuminuria in the subjects;
   b. The risk of cardiovascular events was increased in those with micro-/macroalbuminuria; and
   c. All-cause mortality was increased in the subjects with macroalbuminuria as well as those with normoalbuminuria and microalbuminuria who exhibited a GFR of less than 30 mL/min/1.73 m^2.

While that study was not a true prospective study and involved only a limited number of facilities and patients from a population known to be less prone to cardiovascular

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events than those in Western countries, the findings provide important insight into the prognosis of diabetic nephropathy in Japanese patients. Therefore, in seeking to revise the Classification, the Committee gave due consideration to the above findings. At the same time, the following considerations were also taken into account.

1. The bulk of evidence for the classification of diabetic nephropathy comes from randomized controlled studies enrolling patients with diabetic nephropathy as defined based on the extent of albuminuria, and very little evidence is available for diabetic nephropathy as defined based on GFR.
2. The current "Medical Service Fee Schedule for Guidance on Preventing Diabetes-Associated Dialysis" was developed with the Classification in mind.
3. The "Guidelines for Clinical Efficacy Evaluation of Pharmacological Agents for Diabetic Nephropathy (Draft)" currently in use were developed with the Classification in mind.

Therefore, after giving due consideration to all of these issues during the course of several sessions, the Committee decided to leave the Classification essentially unchanged for now (Table 1), while showing how it may be aligned with the widespread CKD classification based on GFR (eGFR) ("see Appendix"). The former is not, however, presented as a heat map, due to the limitations of the study referred to above, which involved a small number of patients with diabetic nephropathy and included no dialysis patients, providing the basis for this revision. Again, as all kidney diseases affecting patients with diabetes are covered in the Classification, the Committee called for attention with notes included which were required, in order to highlight the importance of the differential diagnosis between diabetic nephropathy and non-diabetic kidney disease in all stages. The differential diagnosis calls for collaboration with nephrologists; such collaboration is not limited to cases requiring a renal biopsy. Furthermore, given that the disease may not always progress in some patients, numerous notes were included in the table in order to call attention to these cases. Additionally, in view of the potential need to use multiple anti-diabetic drugs over time, "Key Precautions in View of Drug Use" are included below the table. The major revisions to the Classification are summarized below:

1. eGFR is now substituted for GFR in the Classification.

Table 1 Classification of Diabetic Nephropathy 2014

Stage	Urinary albumin (mg/g Cr) or urinary protein (g/g Cr)	GFR (eGFR) (mL/min/1.73 m^2)
Stage 1 (pre-nephropathy)	Normoalbuminuria (< 30)	≥30[a]
Stage 2 (incipient nephropathy)	Microalbuminuria (30–299)[b]	≥30
Stage 3 (overt nephropathy)	Macroalbuminuria (≥ 300) or Persistent proteinuria (≥ 0.5)	≥30[c]
Stage 4 (kidney failure)	Any albuminuria/proteinuria status[d]	<30
Stage 5 (dialysis therapy)	Any status on continued dialysis therapy	

Diabetic nephropathy does not always progress from one stage to the next. The revised classification takes into account findings on the prognosis of type 2 diabetic patients from a "historical cohort study" conducted as part of the MHLW-subsidized Project on Kidney Disease, entitled "Diabetic Nephropathy Research, from the Ministry of Health, Labour and Welfare of Japan" [10, 11]

[a] While a GFR of less than 60 mL/min/1.73 m^2 is consistent with the diagnosis of CKD, underlying causes other than diabetic nephropathy may be involved in patients with a GFR below 60 mL/min/1.73 m^2 thus calling for the differential diagnosis between diabetic nephropathy and any other potential non-diabetic kidney diseases

[b] Patients with microalbuminuria are to be diagnosed as incipient nephropathy after the differential diagnosis based on the criteria for an early diagnosis of diabetic nephropathy

[c] Precautions are required in patients with macroalbuminuria, in whom renal events (e.g., a decrease in eGFR to half its baseline value, the need for dialysis) have been shown to increase as the GFR decreases below 60 mL/min/1.73 m^2

[d] All patients with a GFR of less than 30 mL/min/1.73 m^2 are classified as exhibiting kidney failure, regardless of their urinary albumin/protein values. However, in those with normoalbuminuria and microalbuminuria, the differential diagnosis is required between diabetic nephropathy and any other potential non-diabetic kidney diseases

Key Precautions in View of Drug Use: This table is intended, first and foremost, as a classification of diabetic nephropathy and not as a guide to drug use. All drugs, including anti-diabetic drugs, particularly renally metabolized agents, are to be used in accordance with their prescribing information, with due consideration to relevant factors such as GFR in each patient

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2. The stages used in the Classification have been simplified to include normoalbuminuria, microalbuminuria, macroalbuminuria and kidney failure.
3. The division between A and B (early versus late macroalbuminuria) in stage 3 has been abandoned and A and B have been reintegrated, due to the paucity of evidence for proteinuria of 1 g/day as the threshold for dividing the stage.
4. Kidney failure has been redefined in all cases as a GFR less than 30 mL/min/1.73 m^2, which represents the threshold value for kidney failure obtained by quantifying the existing definition of kidney failure in the Classification based on the Classification of the Japanese Society of Nephrology (JSN) [12] with all other pre-kidney failure conditions redefined as a GFR of 30 mL/min/1.73 m^2 or greater.
5. Qualifying or illustrating phases in parentheses, such as "e.g., incipient nephropathy", have been retained throughout the Classification, as they have become common currency in the field, although their removal from the Classification was suggested during the process of revision.
6. Stress is now placed on the differential diagnosis of diabetic nephropathy versus non-diabetic kidney disease as being crucial in all stages of diabetic nephropathy.

Of note, the American Diabetes Association (ADA) proposed in its Clinical Practice Recommendations 2013 that all cases of albuminuria of 30 μg/mg Cr (=mg/g Cr) be defined as "increased urinary albumin excretion", thus abandoning the division between micro- and macroalbuminuria [13]. Again, while this concept was retained in the Clinical Practice Recommendations 2014, the ADA further proposed that microalbuminuria and macroalbuminuria be redefined as persistent albuminuria of 30–299 mg/24 h and ≥300 mg/24 h, respectively [14]. While this change may result in the terms micro- and macroalbuminuria ceasing to be common currency in the clinical setting in the US, to avoid confusion, the Committee has chosen not to follow suit and rather err on the side of caution, thereby retaining these terms in the Classification, given that they are less likely to no longer be used in scientific publications and are expected to remain common currency in Japan.

Last but not least, with a number of multicenter prospective studies currently underway, including the Japan Diabetes Complication and Prevention prospective (JDCP) study, JSN registries, Japan Diabetes Clinical Data Management (JDDM) studies and Japan Diabetes Optimal Integrated Treatment for 3 Major Risk Factors of Cardiovascular Diseases (J-DOIT3) randomized study, the Committee also plans to further revise the Classification in a timely fashion as required, as relevant evidence becomes available from these and other studies.

Conclusions

In order to resolve the discrepancy between the existing Classification of Diabetic Nephropathy and the current Classification of CKD stages, the Joint Committee on Diabetic Nephropathy revised its Classification of Diabetic Nephropathy. The new classification has already been uploaded onto the website of each member society represented on the Joint Committee as of January 10, 2014. Again, in view of further revisions in the years to come, the Joint Committee has termed the revised classification as the "Classification of Diabetic Nephropathy 2014."

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Human rights statement and Informed consent This article does not contain any studies with human or animal subjects performed by the any of the authors.

Appendix

Relationship between the 2014 categories for diabetic nephropathy stages and the CKD severity categories

	Albuminuria category	A1	A2	A3
	Quantitative urinary albumin estimation Urinary albumin/Cr ratio (mg/g Cr) (quantitative urinary protein estimation) (urinary protein/Cr ratio (g/g Cr)	Normoalbuminuria < 30	Microalbuminuria 30-299	Macroalbuminuria ≥300 (or increased proteinuria) (≥0.50)
GFR category (mL/min/1.73 m^2)	≥ 90 60-89 45-59 30-44	Stage 1 (pre-nephropathy)	Stage 2 (incipient nephropathy)	Stage 3 (overt nephropathy)
	15-29 < 15	Stage 4 (kidney failure)		
	(dialysis therapy)	Stage 5 (dialysis therapy)		

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Pro-inflammatory/Th1 gene expression shift in high glucose stimulated mesangial cells and tubular epithelial cells

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ABSTRACT

Diabetic nephropathy (DN) is a major cause of end stage kidney disease and a strong risk factor for cardiovascular diseases. Growing data show chronic inflammation plays an important role for the progression of DN. As for the immune cells, anti-inflammatory leukocytes as well as pro-inflammatory leukocytes play an important role in DN. In addition to leukocytes, renal resident cells contribute to the inflammatory process of DN. However, precise functions, phenotypes and immune balance of renal resident cells remain to be explored. Therefore, we hypothesized that the aberrant immune balance of renal resident cells contributes to the pathogenesis of DN. To explore this possibility, we performed genome-wide transcriptome profiling in mesangial cells and tubular epithelial cells (TECs), which were stimulated by high glucose (HG) and detected the expression of inflammation associated genes. HG increased the mRNA expression of oxidative stress, inflammasome and mammalian target of rapamycin (mTOR) related genes in mesangial cells. Pro-inflammatory/Th1 gene expression was upregulated, but Th2 related gene expression was downregulated in mesangial cells. In TECs, HG stimulation increased pro-inflammatory/Th1/Th2 gene expression. Phosphorylation of signaling proteins shifted towards pro-inflammatory phenotype with suppressed phosphorylation of Th2 related signaling in TECs. The data taken together indicate that HG shifts the immune balance toward pro-inflammatory/Th1 phenotype in mesangial cells and TECs, which might initiate and/or prolong inflammation, thereby resulting in diabetic nephropathy.

1. Introduction

The number of patients with end stage kidney diseases (ESKD) is increasing worldwide. Especially, diabetic nephropathy (DN) is one of the major causes of ESKD in developed countries. Not only the high incidence of ESKD, but also the high mortality from cardiovascular diseases has been observed in patients with DN [1]. Therefore, identifying molecular mechanisms that mediate the progression of DN is an important issue to uncover novel therapeutic targets.

Growing evidence suggests that chronic inflammation plays key roles for the progression of DN [2–4]. Recent studies revealed that macrophages (Mϕ) and T cells contribute to the pathogenesis of DN in both rodent and human models. Activated Mϕ and T cells induce and aggravate the kidney injury, resulting in chronic kidney failure. Recently, Mϕ are categorized as inflammatory (M1) and anti-inflammatory (M2) phenotype according to the cytokine profile and cell surface marker [5]. M2 Mϕ as well as M1 Mϕ has been reported to contribute to the pathogenesis of DN [6–8]. $CD4^+$ T cell populations are also broadly and simplistically divided into 4 types, Th1, Th2, Th17 and regulatory T cells based on the function [9]. Each phenotype of Th cell has been reported as a key player for the progression of DN [10–12]. These data suggest that the immune balance of inflammatory cells affect the prognosis of DN.

Not only accumulated leukocytes, but also renal resident cells orchestrate in the inflammatory processes in DN. Activated renal resident cells secret various cytokines/chemokines [13,14]. However, precise functions, phenotypes and immune balance of renal resident cells remain to be explored in DN. Therefore, we hypothesized that the aberrant immune balance of renal resident cells

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may contribute to the pathogenesis of DM. To explore this hypothesis, we performed genome-wide transcriptome profiling in mesangial cells and tubular epithelial cells (TECs), which were stimulated by high glucose (HG), and identified the expression of inflammatory associated genes.

2. Materials and methods

2.1. Cell culture

Normal human mesangial cells (Lonza, Basel, Switzerland) were cultured in mesangial culture kit (Lonza). Human immortalized TECs, HK-2 (ATCC, Manassas, VA) was cultured in 10% FBS DMEM (Life technologies, Tokyo) with 100 μg/ml streptomycin and 100 U/ml penicillin. All the cells were grown in a humidified atmosphere (5% CO^2/95% air) at 37 °C and were seeded onto six-well cell culture cluster (Corning Incorporated, Corning, NY). The cells were stimulated with 30 mM glucose (Wako, Tokyo) for 24 h for hyperglycemic stimulation. For osmotic control, the cells were cultured with 30 mM mannitol (Wako) for 24 h.

2.2. SAGE analysis using Ion Torrent PGM™

Gene expression analysis was performed by SAGE method and NGS. Briefly, RNA was isolated using mRNA Isolation kit (Sigma–Aldrich, St. louis, MO). NGS data from the PGM™ was generated from 1 μg of total RNA isolated from cell line. SAGE libraries were constructed using the SOLiD SAGE™ kit from Life Technologies (Life Technologies) according to manufacturer's protocol. DNA was recovered from the agarose gel using PureLink Gel Extraction kit (Life Technologies). DNA fragments of SAGE construct were analyzed on the Agilent Bioanalyzer using the High Sensitivity Kit (Agilent, Santa Clara, CA). Template preparation and emulsion PCR, and Ion Sphere Particles (ISP) enrichment was done using the Ion Xpress Template kit (Life Technologies) according to the manufacturer's instructions. The quality of the resultant ISPs was assessed using Qubit 2.0 Fluorometer (Life Technologies), and were loaded and sequenced on a 318 chip (Life Technologies). The complete data sets from these experiments have been deposited in the NCBI Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo; accession number GSE52734).

2.3. Mapping from NGS data

For each of the samples, the PGM™ raw reads were aligned against the human refseq genes (UCSC, http/hgdownload.cse.ucsc.edu/) using BWA 0.6.2 which uses the 25_1 mapping parameter. We generated unique gene counts by excluding reads that mapped to contigs of more than one gene. Reads mapping to several contigs within an isogroup were only counted once.

2.4. Antibodies

The following antibodies were used for flow cytometric analysis; Phycoerythrin (PE) conjugated anti-phosphorylated p38 (612565; BD Biosciences, San Jose, CA), PE-conjugated anti-phosphorylated STAT1 (562674; BD Biosciences), PE-conjugated anti-phosphorylated STAT3 (558557; BD Biosciences), PE-conjugated anti-phosphorylated STAT5 (61257; BD Biosciences), PE-conjugated anti-phosphorylated STAT6 (612701; BD Biosciences).

2.5. Flow cytometric analysis

To detect phosphorylated intracellular signaling, cells were fixed and permeabilized using BD Phosflow™ in accordance with the manufacturer's protocol (BD Biosciences). Then, the cells were stained with antibody. We collected 10,000 cultured cells using FACSCalibur (BD Biosciences) and analyzed data using FlowJo software 9.3 (Tree Star, Palo Alto, CA).

2.6. Reconstitution of signal transduction pathway

To identify the signal transduction pathway, each gene was reconstituted according to Kyoto Encyclopedia of Genes and Genomes (KEGG: http://www.genome.jp/kegg/) database.

2.7. Statistics

Statistical analysis was performed as described before [15]. Data represent the expression ratio (HG stimulated/osmotic control).

3. Result

3.1. High glucose (HG) increased the mRNA expression of oxidative stress, inflammasome and mammalian target of rapamycin (mTOR) related genes

Oxidative stress [16,17] has been regarded as intracellular mediator in HG induced inflammation. Thus, we examined the mRNA expression of oxidative stress associated genes. Among them, reactive oxygen species modulater 1 (ROMO) and protein kinase C (PKC) alpha (PRKCA) mRNA were increased in HG stimulated mesangial cells (Fig. 1). Recent study revealed that PKC signaling is closely related to mTOR signaling [18,19]. Therefore, we analyzed mTOR signaling related genes. Interestingly, the relative gene expression of mTOR and its downstream signaling, translation initiation factor 4B (EIF4B) were upregulated. Moreover, thioredoxin interacting protein (TXNIP) mRNA, which has the potential to activate the NOD-like receptor family, pyrin domain containing (NLRP) 3 inflammasome [20], was also increased in HG stimulated mesangial cell. In addition, transcription factor, activated protein (AP)-1 (JUN, FOSL2), C–C chemokine ligand (CCL) 2 and tumor necrosis factor (TNF)-α mRNA were also increased higher in HG stimulated mesangial cells as compared to osmotic control.

3.2. HG stimulated mesangial cells were skewed toward an inflammatory phenotype

As HG increased the mRNA expression levels of inflammatory signal mediators, we look into whether HG stimulation shifted the cell phenotype toward an inflammatory population. Inflammatory cytokines/chemokines and their receptors, such as CCL2, TNF-α and interleukin(IL)-1 receptor mRNA expression were increased by the HG stimulation in mesangial cells. Moreover, mitogen activated protein kinase (MAPK) mRNA expression was also increased in HG stimulated mesangial cells. In contrast, Th2 related cytokine receptors and intracellular signaling molecules showed the reduced mRNA expression levels (Table 1). Taken together, HG stimulation increased mRNA expression of the pro-inflammatory molecules and decreased Th2 related molecules in mesangial cells.

3.3. Increased mRNA expression of inflammatory cytokines/chemokines and Th2 type cytokine receptors in HG stimulated TECs

Next, we examined the functional phenotype of HG stimulated TECs. Stimulated TECs showed the increased mRMA levels of inflammatory cytokines/chemokines and their receptors, such as TNF-α, IL-1 receptor, IL-18, IL-12 and IL-6. In addition, mRNA expression of Th2 related cytokine receptors, IL-10 receptor and IL-13 receptor, also increased via HG stimulation (Table. 2).

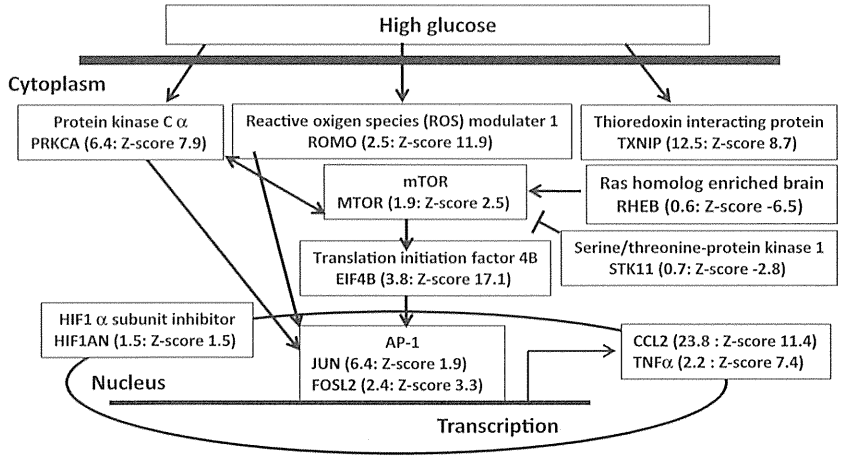

Fig. 1. High glucose (HG) increased the mRNA expression of oxidative stress, inflammasome and mammalian target of rapamycin (mTOR) related genes. HG increased the mRNA expression of reactive oxygen species modulater 1 (ROMO), protein kinase C alpha (PRKCA) and thioredoxin interacting protein (TXNIP). Moreover, gene expression of mTOR and its downstream signaling, translation initiation factor 4B (EIF4B) were also upregulated. Data represents the ratio of gene expression (HG stimulated mesangial cells/mannitol treated mesangial cells).

Table 1
HG stimulated mesangial cells were skewed toward an inflammatory phenotype.

	Increased genes ↑ (High glucose/mannitol control)		Decreased genes ↓ (High glucose/mannitol control)	
Pro-inflammatory	CCL2 (23.8)	MAPK1 (1.6)		MAPK7 (0.7)
	TNFα (2.2)	MAPK3 (1.1)		
	IL-1R (2.0)	MAPK6 (1.7)		
Th1		STAT1 (1.3)	IFN-γR (0.5)	
Th2			IL-4R (0.5)	STAT5 (0.5)
			IL-10Rβ (0.9)	STAT6 (0.5)
			IL-13Rβ (0.8)	
Th17	IL-6ST (1.3)		IL-6 (0.5)	STAT3 (0.3)
TGF		SMAD1 (1.3)		SMAD3 (0.4)
		SMAD2 (1.7)		

Abbreviations are CCL: CC chemokine ligand, TNF: tumor necrosis factor, MAPK: mitogen activated protein kinase, STAT: signal transducer and activator of transcription, IFN: interferon, IL: interleukin, SMAD: Sma and Mad related family.

Table 2
Increased mRNA expression of inflammatory cytokine/chemokine and Th2 type cytokine receptor in HG stimulated TECs.

	Increased genes ↑ (High glucose/mannitol control)		Decreased genes ↓ (High glucose/mannitol control)	
Pro-inflammatory	TNFα (1.5)	MAPK1 (1.3)		MAPK7 (0.6)
	IL-1R (3.0)			
Th1	IL-18 (1.8)	STAT1 (2.8)	IFN-γR (0.8)	
	IL-12 (2.0)			
Th2	IL-10Rβ (1.7)			
	IL-13Rα (2.0)			
Th17	IL-6 (3.6)			
TGF				TGF-β1 (0.8)

Abbreviations are TNF: tumor necrosis factor, MAPK: mitogen activated protein kinase, STAT: signal transducer and activator of transcription, IFN: interferon, IL: interleukin, TGF: transforming growth factor.

3.4. Phosphorylation of intracellular signaling molecules were increased in HG stimulated mesangial cells and TECs

Intracellular signaling has crucial roles in cytokines/chemokines- mediated cellular reactions, such as proliferation, differentiation and activation. The phosphorylation of signaling proteins is essential for the activation of the signal transduction pathways. Therefore, we examined the phosphorylation of signaling proteins in HG stimulated mesangial cells and TECs. Phosphorylated form of p38 MAPK and signal transducer and activator of transcription (STAT) 3, which are related to pro-inflammatory/Th1 signaling [21] were significantly higher in 24 h HG stimulated mesangial cells and TECs. Conversely, phosphorylated forms of STAT5 and STAT6, which are associated with Th2 type signaling transductions [21], were significantly lower in 24 h HG stimulated TECs (Fig. 2). These data suggest the shift of the immune balance toward pro-inflammatory phenotype in mesangial cells and TECs stimulated with HG.

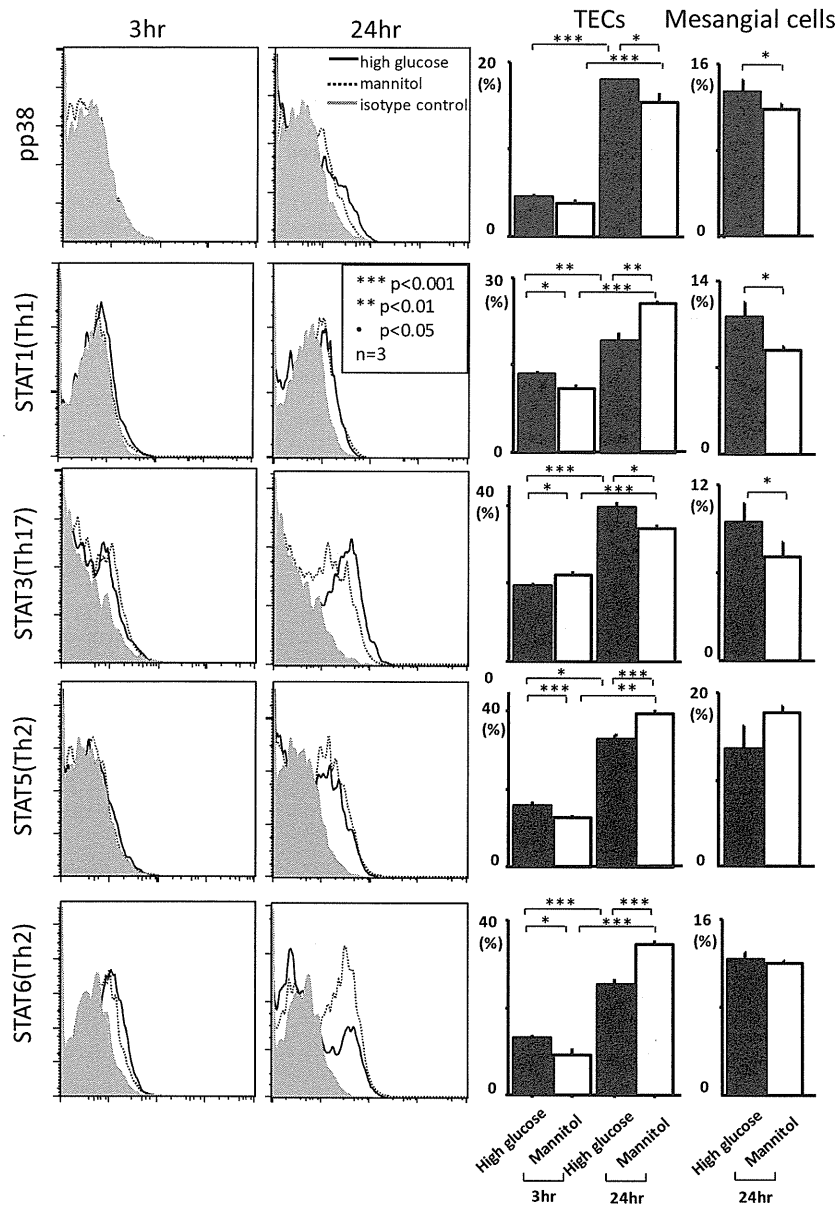

Fig. 2. Pro-inflammatory/Th17 related phosphorylated intracellular signaling were increased in HG stimulated mesangial cells and TECs. Phosphorylated p38 MAPK and STAT3 was significantly higher in 24 h HG stimulated mesangial cells and TECs. Conversely, phosphorylated STAT5 and STAT6 were significantly lower in 24 h HG stimulated TECs. Data represents means ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

4. Discussion

We now report that HG stimulation increases pro-inflammatory/Th1 gene expression, but decreases Th2 related gene expression in mesangial cells. In TECs, HG stimulation increased pro-inflammatory/Th1/Th2 gene expression. Phosphorylation of signaling proteins shifted towards pro-inflammatory phenotype with suppressed phosphorylation of Th2 related signaling proteins in mesangial cells and TECs. The data taken together indicate that HG shifts the immune balance toward pro-inflammaotry/Th1 phenotype in mesangial cells and TECs, which might initiate and/or prolong inflammation, thereby resulting in diabetic nephropathy.

Growing evidence suggests that chronic inflammation plays important roles in the progression of DN [2–4]. Not only the leukocytes, but also renal resident cells, including mesangial cells and TECs participate in the pathogenesis of DN. HG stimulation induced the gene expression of PKC and ROS related proteins in mesangial cells. PKC and ROS related proteins have been reported as the key signaling for HG induced mesangial cell damage [16,17]. Therefore, our data suggest HG stimulation activated intracellular signaling pathway in mesangial cells. Interestingly, the relative gene expression of mTOR and its downstream signaling, translation initiation factor 4B (EIF4B) were upregulated. Supporting this notion, the recent study revealed that PKC signaling is closely related to mTOR signaling [18,19]. Moreover, mTOR expression has been reported in mesangial cells and podocytes in DN [22,23]. In addition, thioredoxin interacting protein (TXNIP) gene expression was also increased in HG stimulated mesangial cells. Lerner et al. reported that increased TXNIP activate NLRP 3 inflammasome, causing procaspase-1 cleavage and IL-1β secretion [20]. Another group also reported the involvement of inflammasome in DN [24]. Thus, our data suggest HG stimulation activate mTOR signaling and

inflammasome in cultured mesangial cells. However, more precise analysis are needed to confirm if these signaling pathway is really involved in DN.

Recently, Mϕ are categorized as inflammatory (M1) and anti-inflammatory (M2) phenotype according to the cytokine profile and cell surface markers [5]. $CD4^+$ T cell populations are also divided into 4 types, Th1, Th2, Th17 and regulatory T cells based on the function [9]. Orchestration of inflammation by pro-inflammatory cells with anti-inflammatory cells has an impact on the process of progressive kidney diseases including DN. In this study, mesangial cells and TECs displayed pro-inflammatory phenotype with the reduction of Th2 related genes via HG stimulation. In support of our findings, Min et al. reported that HG increases TNF-α and IL-6 secretion in mesangial cells [25]. Also tubular epithelial cells have been reported as an important source for cytokines/chemokines in diabetes [14]. Moreover, suppression of JAK/STAT signaling decreased the expression of pro-inflammatory cytokines/chemokines, resulting in improved kidney injury in a rat diabetes model [26]. In addition, we have reported that repairing TECs from hypoxia injury releases mediator that skews M1 Mϕ toward M2 phenotype [27]. The data taken together indicate that renal resident cells such as mesangial cells and TECs orchestrate in the inflammatory processes with changing the immune balance.

As for the intracellular signaling pathway, phosphorylation plays critical roles in signal transduction. Thus, we analyzed the phosphorylation of each molecule. HG stimulation for 24 h increased the phosphorylation of p38 and STAT3. P38 MAPK is a key signaling for inflammatory cytokines/chemokines, thereby contributing to the progression of inflammatory kidney diseases [28–31], including DN [8]. STAT3 pathway is required for IL-23/IL-17 signaling, thereby leading helper T cell to Th17 axis [21]. Supporting our notion, STAT3 expression has been reported in mesangial cell [32], mediating cell proliferation and activation. Moreover, Ranganathan et al. reported the increased collagen expression via STAT3 activation in TECs [33]. However, the association STAT3 and Th17 axis is not clear in renal resident cells. Loverre et al. reported the IL-17 expression in TECs in renal transplant recipients with acute rejection, though STAT3 activation was not mentioned [34].

In contrast, STAT5 and STAT6 phosphorylation were decreased in HG stimulated TEC. IL-2/STAT5 and IL-4/STAT6 signalings promote Th2 polarization in CD4 helper T cell [21,35]. Interestingly, STAT6 signaling showed reno-protective potential in crescentic glomerulonephritis [36] and ischemia–reperfusion (I/R) injury [37]. Moreover, STAT5 signaling plays a central role in erythropoietin mediated tissue protection in I/R injury [38] and cisplatin induced acute kidney injury [39]. Therefore, the reduction of STAT6 and STAT5 signaling might suggest the blunted protective effect in HG stimulated TECs. However, the precise role of STAT5 and STAT6 signalings in DN remains to be investigated.

In conclusion, HG stimulation changes the immune balance toward inflammatory phenotype in mesangial cells and TECs, which might initiate and/or prolong inflammation, thereby resulting in DN. We anticipate that future studies elucidate the precise mechanisms of aberrant immune balance and thereby provide novel therapeutic approaches to DN.

Conflict of interest

The authors have declared that no conflict of interest exists.

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