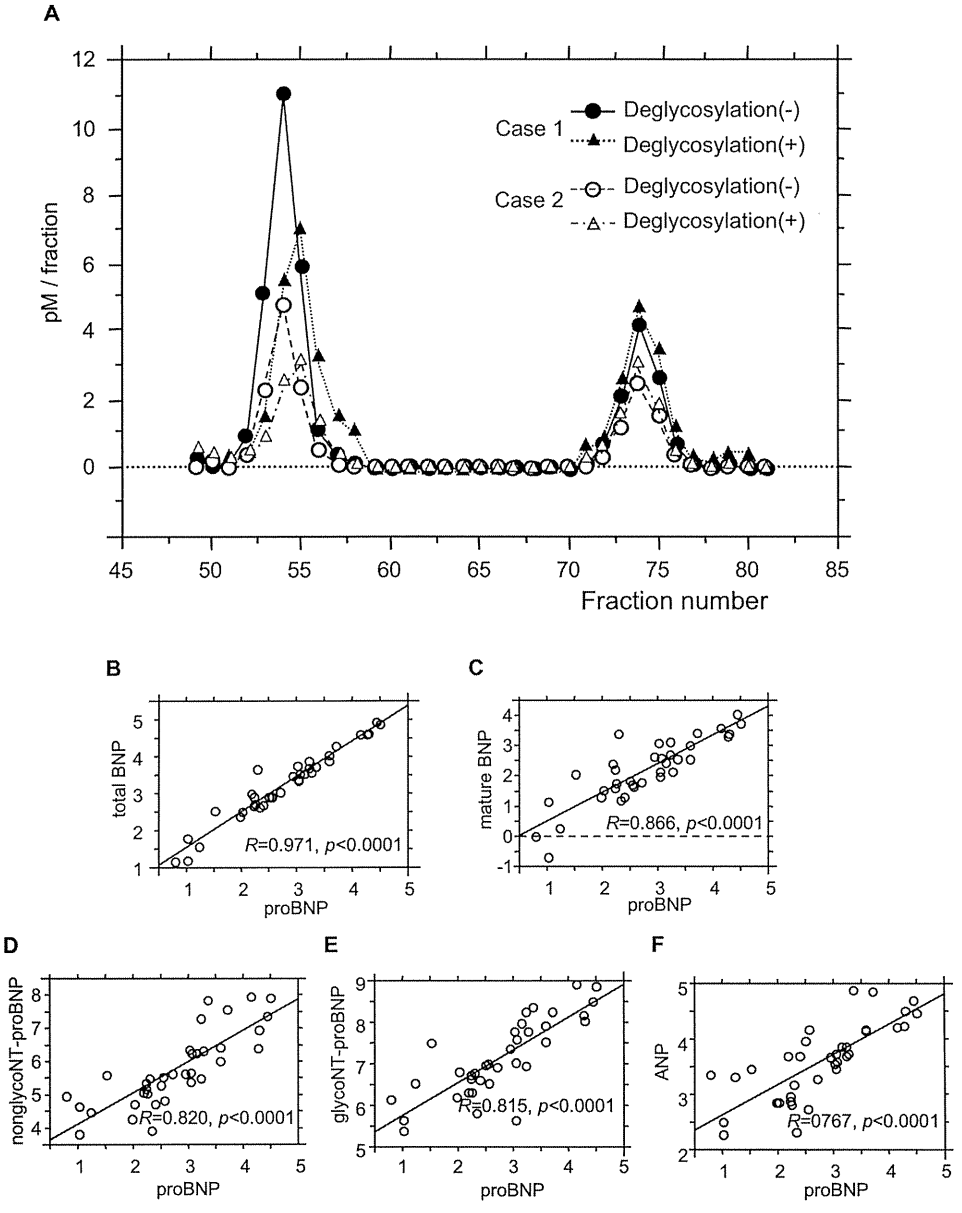

Figure 2. Gel filtration analysis of total BNP and proBNP in hemodialysis patients. (A) Fractions were assayed using total BNP systems. The first and second peaks show proBNP and BNP-32, respectively. (B–F) Correlations between proBNP and other natriuretic peptide-related molecules in ESRD patients before hemodialysis. *R* is the correlation coefficient. Values are expressed as $\log_{10}$ of each level of the indicated molecules.
doi:10.1371/journal.pone.0092314.g002

proBNP, glycoNT-proBNP, ANP and cGMP, in healthy subjects and ESRD patients, and found that all of them were higher in ESRD patients, most of whom had preserved cardiac systolic function (Table 1), than in healthy persons. These increases in ESRD patients can be explained by reductions in their clearance due to the renal failure, increased plasma volume, and diastolic dysfunction caused by left ventricular hypertrophy, among others. Moreover we also found that all of these molecules declined during hemodialysis, though the magnitudes of the reductions did not correlate with any indices of body fluid volume. These results may be explained in part by dialyzer membrane-mediated removal. The so-called super-flux membranes currently in use are able to remove medium-sized molecular solutes, such as β2-microglobulin (MW = 12,000), which enables removal of larger and protein-bound uremic toxins. The pore size for the membranes used in the present study was 78–84 Å, and the sieving coefficient for β2-microglobulin was 0.99. It is therefore likely that the super-flux membranes removed a great deal of BNP and BNP-related molecules during hemodialysis.

Consistent with that idea, the reduction ratio for cGMP, which has a molecular weight of about 500, was the largest, while the reduction ratio for proBNP, which has a molecular weight of about 20,000~25,000, was the smallest. The reduction ratio for mature BNP (MW: 3,500) was similar to that of ANP (MW 3,000), reflecting the similarity of their molecular weights. The reduction ratio for total BNP was comparatively modest, because it is the mean of the ratios for mature BNP and proBNP. It thus appears that the magnitudes of the reductions in peptide concentration associated with hemodialysis depends on their molecular weights.

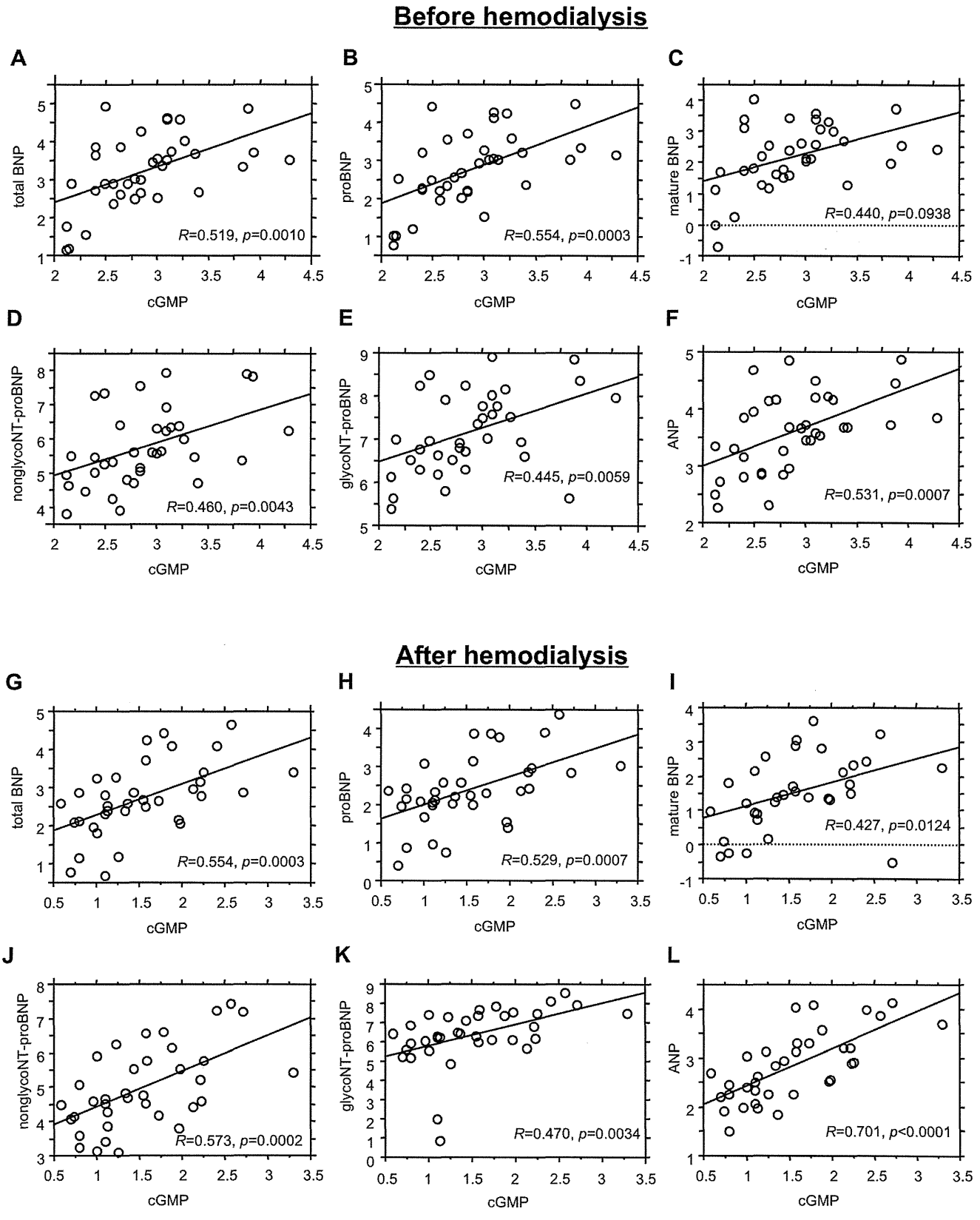

Figure 3. Correlations between cGMP and natriuretic peptide-related molecules in ESRD patients before and after hemodialysis. *R* is the correlation coefficient. Values are expressed as $\log_{10}$ of each level of the indicated molecules.
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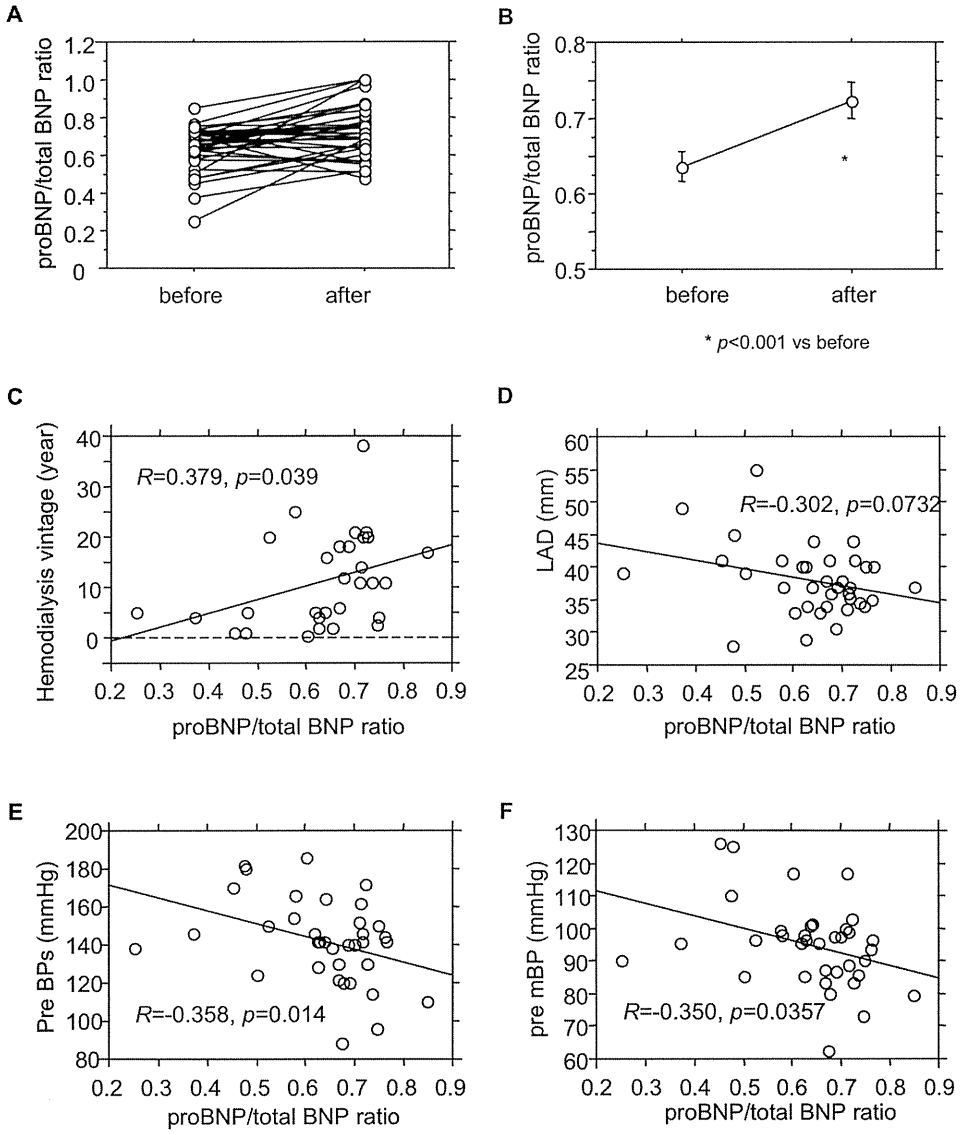

Figure 4. ProBNP/total BNP ratios and their correlation with the indicated clinical parameters. (A) Individual changes in proBNP/total BNP ratios in ESRD patients during hemodialysis: Before, before hemodialysis; After, after hemodialysis. (B) ProBNP/total BNP ratios in ESRD patients during hemodialysis expressed as means ± SE. (C–F) Correlation between proBNP/total BN ratios and hemodialysis vintage (C), left atrial diameter (LAD) (D), systolic blood pressure before hemodialysis (preBPs) (E) and mean blood pressure before hemodialysis (pre mBP) (F). *R* is the correlation coefficient between the indicated parameters.
doi:10.1371/journal.pone.0092314.g004

Previously, we also reported the plasma levels of BNP, nonglycoNT-proBNP, glycoNT-proBNP, ANP and cGMP in patients with chronic renal failure before and after hemodialysis. [14] In that study, patients were hemodialyzed using older generation dialyzers that removed less of the large solutes like β2-microglobulin than the super-flux membrane we used in the present study. In that earlier study the calculated reduction ratio for each parameter during hemodialysis was as follows: BNP 37.9%, nonglycoNT-proBNP 23.0%, glycoNT-proBNP 4.4%, ANP 61.0%, and cGMP 75.0%. The reduction ratio for nonglycoNT-proBNP and glycoNT-proBNP, which are higher molecular weight molecules, was much smaller than in the present study, most likely due to the difference in the membrane's ability to remove large molecules.

In the present study, we used the Elecsys proBNP II assay (Roche Diagnostics, Indianapolis, IN, USA) to measure non-glycoNT-proBNP and glycoNT-proBNP, with and without deglycosylation enzyme treatment. This assay is the most frequently used NT-proBNP assay in the world and contains a capture monoclonal and a signal monoclonal antibody that recognizes NT-proBNP[27–31] and NT-proBNP[42–46], respectively. Notably, NT-proBNP[42–46] has a glycosylation site at amino acid 44, and a recent study demonstrated that *O*-linked oligosaccharide attachment markedly inhibits binding of the antibody to its antigen peptide [16]. Consequently, Elecsys proBNP II is thought to measure only nonglycoNT-proBNP. We found that nonglycoNT-proBNP was greatly elevated in ESRD patients and that levels of glycoNT-proBNP were 4–5 times higher than those of nonglycoNT-proBNP. The remarkable increase of nonglycoNT-proBNP in hemodialysis patients is thought to reflect the following conditions: (1) NT-proBNP does not bind to the natriuretic peptide receptor-A or -C; (2) NT-proBNP is not

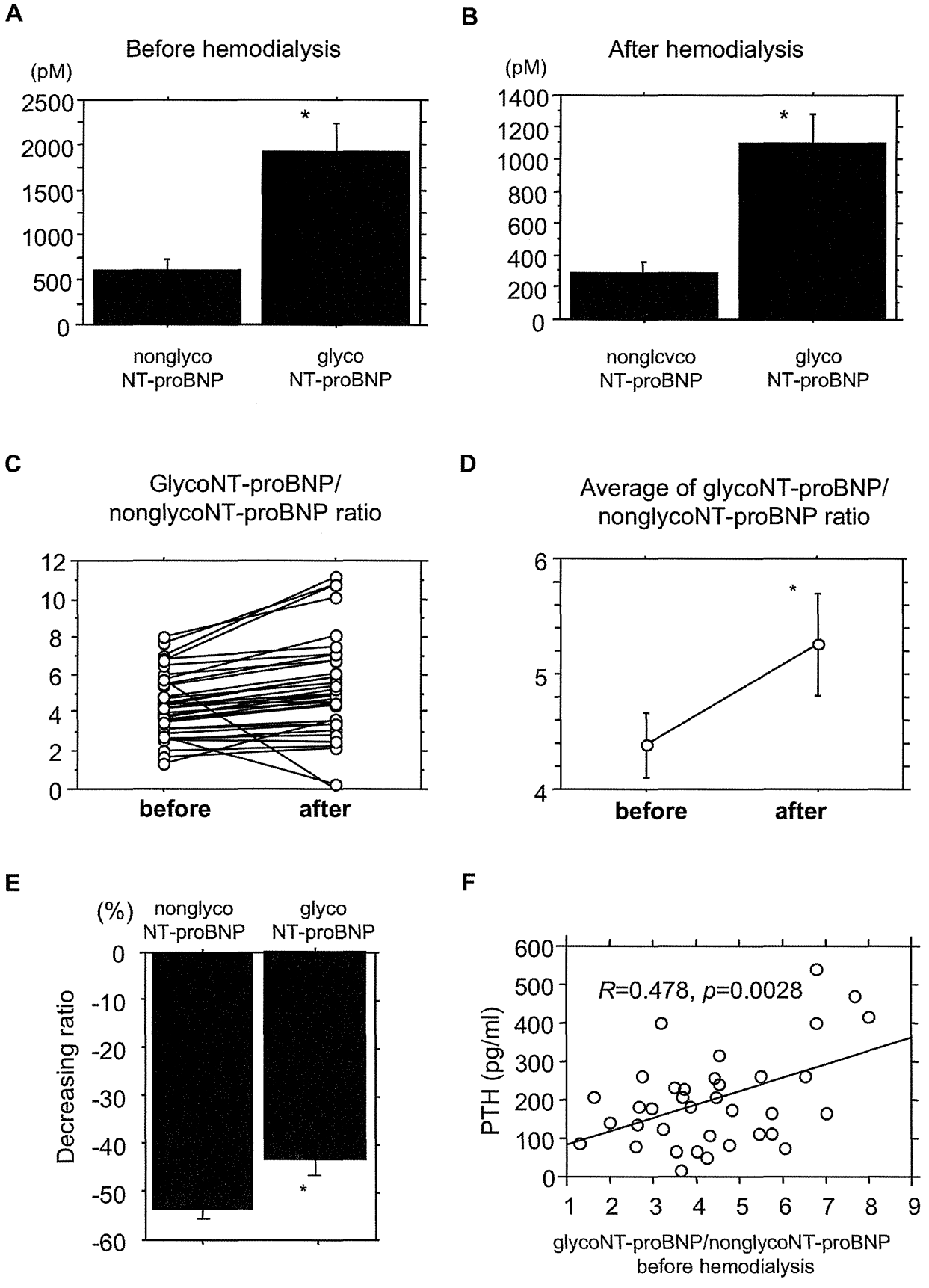

Figure 5. NonglycoNT-proBNP and glycoproBNP levels, glycoNT-proBNP/nonglycoNT-proBNP ratios and correlations between glycoNT-proBNP/nonglycoNT-proBNP ratios and clinical parameters. (A, B) Circulating levels of nonglycoNT-proBNP and glycoNT-proBNP in

ESRD patients before (A) and after (B) hemodialysis. Values are expressed means ± SE. (C, D) Individual changes and values of glycoNT-proBNP/nonglycoNT-proBNP ratios in ESRD patients during hemodialysis. Values are expressed as mean ± SE. *p<0.001 vs. before hemodialysis. before, before hemodialysis; after, after hemodialysis. (E) Reduction ratios for nonglycoNT-proBNP and glycoNT-proBNP during hemodialysis. *p<0.001 vs. nonglycoNT-proBNP. (F) Correlation between the glycoNT-proBNP/nonglycoNT-proBNP ratio and the serum parathyroid hormone (PTH) levels. *R* is the correlation coefficient.
doi:10.1371/journal.pone.0092314.g005

metabolized by neutral endopeptidase; and (3) clearance of NT-proBNP is largely dependent on excretion from the kidney. The glycoNT-proBNP/nonglycoNT-proBNP ratio was larger after hemodialysis than before it. This is most likely because the molecular weight of glycoNT-proBNP is much larger than that of nonglycoNT-proBNP, so it is less likely to be affected by membrane-dependent removal than nonglycoNT-proBNP.

Using gel-filtration HPLC combined with direct chemiluminescent immunoassay, in the present study we showed that the major molecular form of proBNP in the plasma of hemodialysis patients is glycosylated proBNP. Similarly, recent studies have also shown that the major molecular form of plasma proBNP in patients with heart failure and control is glycosylated proBNP [9]. It is thus possible that proBNP in human plasma is glycosylated. We found that proBNP levels were closely correlated with those of total BNP, mature BNP, nonglycoNT-proBNP, glycoNT-proBNP and ANP. This suggests proBNP may also be a useful biomarker of cardiovascular disease, like BNP and NT-proBNP, two well-established biomarkers of cardiovascular disease in ESRD patients. In addition, levels of proBNP correlated significantly with cGMP before hemodialysis, suggesting proBNP may be a good marker of the biological action of natriuretic peptides. After hemodialysis, proBNP still correlated significantly with cGMP, but the correlation coefficient was modest. This may be because the reduction ratio for proBNP is small, while that for cGMP is large.

Interestingly, the proBNP/total BNP ratio correlated positively with hemodialysis vintage and correlated negatively with LAD and blood pressure. Longer hemodialysis vintage may lead to accumulation of the glycosylated proBNP, and diastolic dysfunction and/or increased afterload may alter the processing of glycosylated proBNP to BNP and glycoNT-proBNP. Notably, the glycoNT-proBNP/nonglycoNT-proBNP ratio correlated positively with parathyroid hormone levels in hemodialysis patients. Given that parathyroid hormone induces cardiac hypertrophy via its receptor on myocytes [33], its signal may influence glycosylation within cardiac myocytes and thus the glycoNT-proBNP/nonglycoNT-proBNP ratio. Further studies will be needed to elucidate the mechanism involved.

It has been suggested that hemodialysis reduces natriuretic peptide levels through dialytic clearance or by improving volume control, which results in decreased cardiac overload and reduced secretion from the heart [34]. A limitation of the present study is that we evaluated changes in volume status during hemodialysis based only on changes in body weight or estimated total body fluid volume. No other methods (e.g., bioimpedance analysis) were used. In addition, we did not perform echocardiography after hemodialysis. Had we evaluated changes in cardiac load during hemodialysis using different methods, perhaps some relationship might have been found between changes of the levels of natriuretic peptide-related molecules during hemodialysis and the magnitude of the reduction in cardiac load. Nonetheless, our results suggest that the reduction in the natriuretic peptide-related molecules after hemodialysis is due not only to the reduction in atrial overload, but also to removal via the super-flux dialyzer.

In conclusion, this is a first report showing the hemodialysis-associated changes of natriuretic peptide-related molecules, including proBNP, in ESRD patients in the super-flux dialyzer era. With the development of the super-flux dialysis membrane, there has been a marked change in the kinetics of molecules before and after hemodialysis. In addition, recent studies have shown that glycosylated proBNP is a major molecular form in human plasma and that glycosylated NT-proBNP is underestimated by the NT-proBNP assay system currently being used. Under these conditions, correct interpretation of the peptide levels in the plasma of ESRD patients undergoing hemodialysis and their clinical application may require careful consideration. Further study will be necessary to determine which BNP-related peptides, including proBNP, are most indicative of cardiac complications and predictive of prognosis in hemodialysis patients.

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Author Contributions

Conceived and designed the experiments: YN TN. Performed the experiments: YN TN K. Kuwahara SY HK YK Kazuhiro Nakao TM. Analyzed the data: CY KU YI. Contributed reagents/materials/analysis tools: HO KH K. Nagata K. Kangawa NM. Wrote the paper: TN YN. Supervision: Kazuwa Nakao.

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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

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.

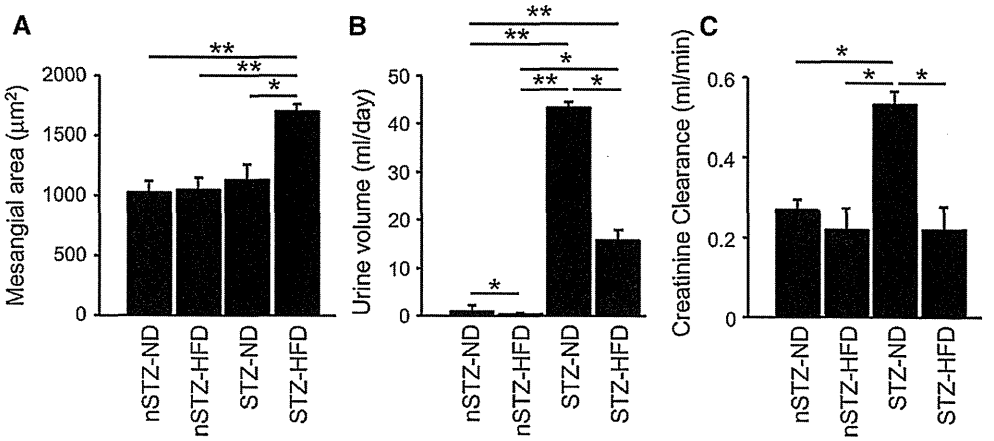

Fig. 1 Effects of STZ and/or HFD upon mesangial expansion (**a**), urine volume (**b**) and creatinine clearance (**c**) in wild-type mice. *nSTZ-ND* non STZ-normal diet, *nSTZ-HFD* non STZ-high fat diet, *STZ-ND* STZ-normal diet, *STZ-HFD* STZ-high fat diet. Data are mean ± SEM. $n = 4$–11. $^{*}p 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

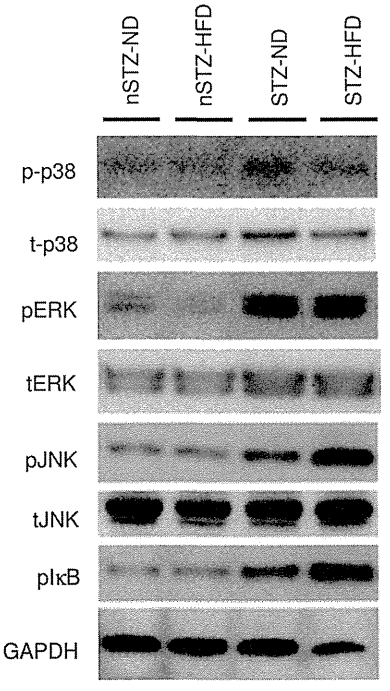

Fig. 2 Western blot analysis for phosphorylation of MAP kinases and IκB in kidney of STZ + HFD mice. *p-/t-p38* phosphorylated/total p38 MAP kinase, *p/tERK* phosphorylated/total extracellular signal-regulated kinase, *p/tJNK* phosphorylated/total c-Jun N-terminal kinase, *pIκB* phosphorylated inhibitor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

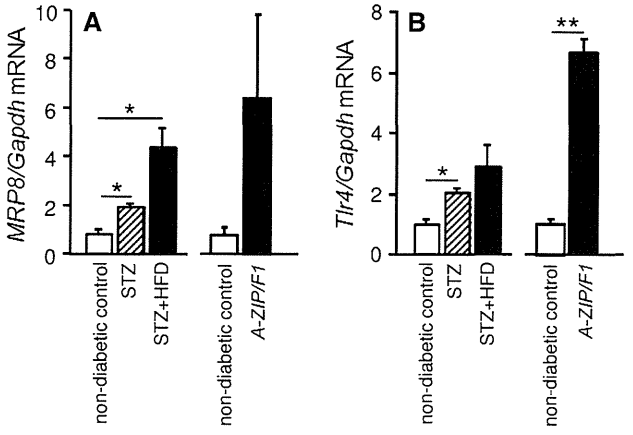

Fig. 3 Glomerular gene expression of *MRP8* (**a**) and *Tlr4* (**b**) in STZ + HFD and lipoatrophic *A-ZIP/F-1* mice determined by Taq-Man real-time PCR. *White bars* non-diabetic control group, *striped bars* diabetic group, *black bars* diabetic-hyperlipidemic group. Data are mean ± SEM. $n = 4$–7.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

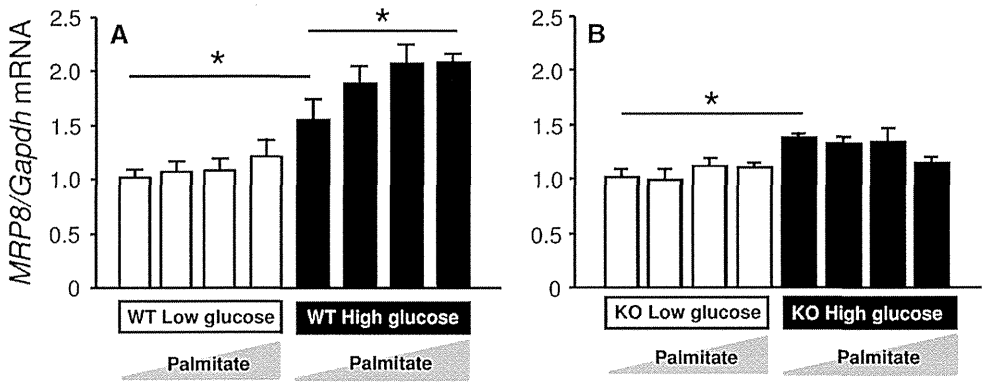

Fig. 4 Gene expression of *MRP8* and effects of glucose or fatty acid in bone marrow-derived macrophages (BMDMs) determined by TaqMan real-time PCR. BMDMs generated from wild-type (WT, **a**) or *Tlr4* knockout (KO, **b**) mice were cultured under low-glucose (100 mg/dl, *white bars*) or high-glucose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

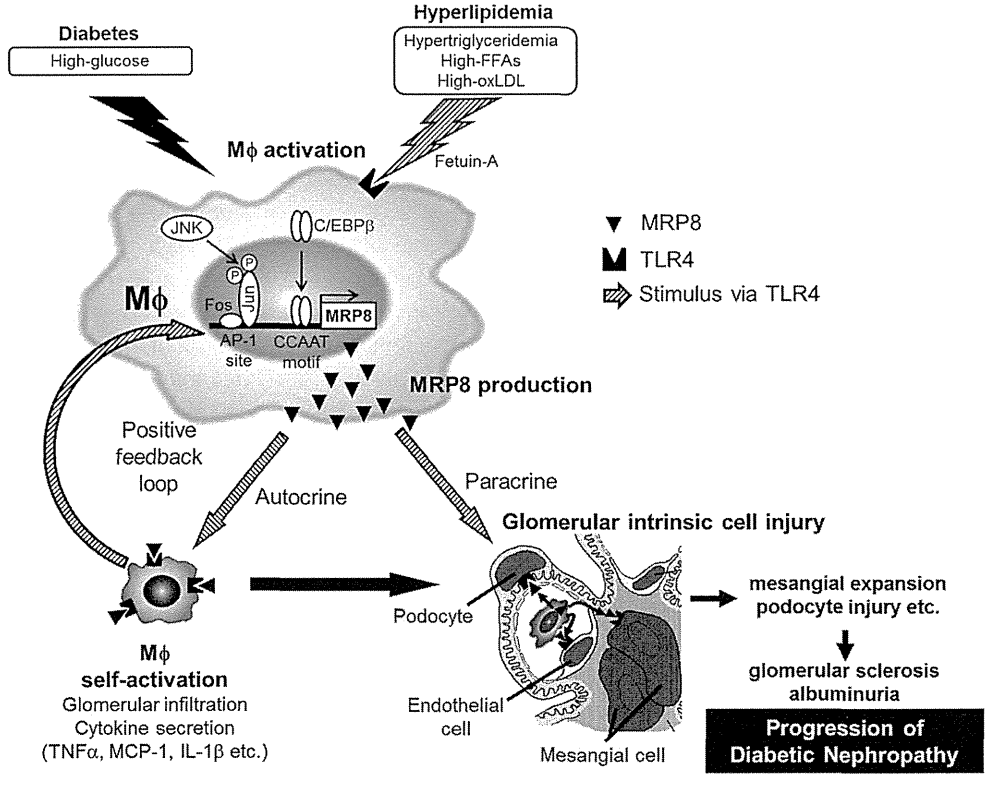

Fig. 5 Proposed mechanism of macrophage-mediated glucolipotoxicity in diabetic nephropathy. Hyperlipidemia (or high free fatty acids) activates circulating macrophages through TLR4-mediated upregulation of MRP8, specifically under hyperglycemic conditions. These synergistic effects upon MRPã8 production 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).

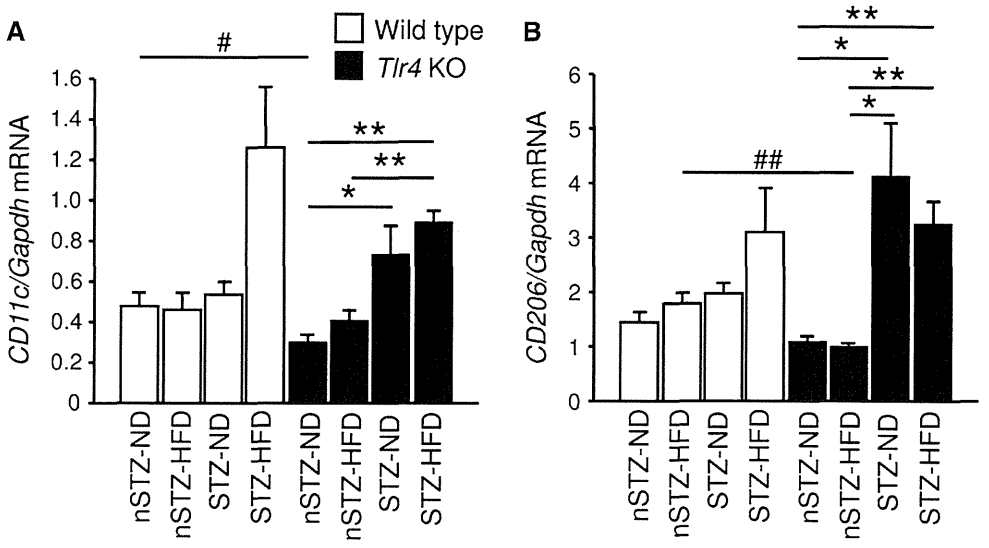

Fig. 6 Glomerular gene expression of M1 (**a**) and M2 (**b**) macrophage markers in STZ-HFD mice determined by TaqMan real-time PCR. Data are mean ± SEM. $n = 4$–11. $^{*}p < 0.05$, $^{**}p < 0.01$. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ for similarly treated *Tlr4* KO versus wild-type

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.

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Conflict of interest The authors have declared no competing interest.

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Predictive Significance of Kidney Myeloid-Related Protein 8 Expression in Patients with Obesity- or Type 2 Diabetes-Associated Kidney Diseases

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Abstract

Background and Objective: We have reported that toll-like receptor 4 (TLR4) and one of its endogenous ligands, myeloid-related protein 8 (MRP8 or S100A8), play an important role in the progression of diabetic nephropathy in mice. The aim of this study was to evaluate significance of kidney MRP8 expression in patients with obesity- or type 2 diabetes-associated kidney diseases.

Methods: In diabetic, obese or control subjects, MRP8 mRNA and protein expression levels in renal biopsy samples were determined by real-time RT-PCR and immunohistochemistry ($n=28$ and 65, respectively), and their associations with baseline and prognostic parameters were analyzed. Effects of MRP8 upon pro-inflammatory gene expressions were examined using macrophages.

Results: Kidney MRP8 gene and protein expression levels were elevated in obese or diabetic groups compared to control group. Among all subjects, by univariate linear regression analysis, glomerular MRP8-positive cell count and tubulointerstitial MRP8-positive area at baseline were both, respectively, correlated not only with various known risk factors for diabetic nephropathy (such as systolic blood pressure, proteinuria and serum creatinine) but also with extent of glomerulosclerosis and tubulointerstitial fibrosis. Independent factors predicting urinary protein levels a year later were examined by multivariate analysis, and they included glomerular MRP8-positive cell count ($\beta=0.59$, $P<0.001$), proteinuria ($\beta=0.37$, $P=0.002$) and systolic blood pressure ($\beta=0.21$, $P=0.04$) at baseline, after adjustment for known risk factors. MRP8 protein expression was observed in CD68-positive macrophages and atrophic tubules. In cultured mouse macrophages, MRP8 protein induced proinflammatory cytokine expressions and also triggered auto-induction of MRP8 in a TLR4-dependent manner.

Conclusions: Glomerular MRP8 expression appears to be associated with progression of proteinuria in obese or type 2 diabetic patients, possibly by inducing inflammatory changes in macrophages through TLR4 signaling.

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Introduction

Chronic inflammation plays an important role in the pathogenesis of diabetes or obesity and their cardiovascular complications [1]. Involvement of innate immune receptors and the endogenous ligands in the process of chronic inflammation has been implicated. Myeloid-related protein 8 (MRP8, also known as S100A8 or calgranulin A) was originally identified as a cytoplasmic calcium-binding protein in neutrophils and monocytes [2], and has become widely recognized as a potent endogenous ligand for toll-like receptor 4 (TLR4) in various diseases including septic shock, vascular and autoimmune disorders [3,4,5]. We have recently proposed that MRP8/TLR4 signaling plays an important role in hyperlipidemia-induced progression of diabetic nephropathy [6]. Glomerular macrophages and collecting duct cells are major sources of MRP8 in mouse models of diabetic nephropathy [6] and renal fibrosis [7], respectively. Plasma levels of MRP8, which usually forms a heterodimeric complex with a binding

partner MRP14 in the bloodstream, are increased in obese subjects [8,9]. However, there have been no reports investigating renal expression of MRP8 in patients with obesity or type 2 diabetes and its association with renal prognosis.

The aim of this study was to determine mRNA and protein expression levels of MRP8 in the kidney of Japanese patients with diabetic nephropathy (DN), obesity-related glomerulopathy (ORG), minimal change nephrotic syndrome (MCNS) or minor glomerular abnormality (MGA), which were all diagnosed by renal biopsy, and to evaluate whether renal MRP8 expression can predict renal outcomes.

Materials and Methods

Ethics statement

The human study was conducted according to the principles expressed in the Declaration of Helsinki, and was approved by the Ethical Committees on Human Research of Kyoto University Graduate School of Medicine and Osaka City General Hospital, respectively. All participants gave written informed consent. The animal study protocol was approved by the Animal Research Committee of Kyoto University Graduate School of Medicine (Permit Number: Med Kyo 13318). All animal surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

Study subjects

Proteinuric patients with obesity or type 2 diabetes who underwent renal biopsy were enrolled in this study. Patients with infectious disease, cancer, liver disease or collagen disease were excluded. Proteinuria was defined as urinary protein greater than 0.5 g/g creatinine or urinary albumin greater than 300 mg/g creatinine in at least two consecutive measurements. Obesity was defined as body mass index (BMI) greater than 25.0 (kg/m^2). Type 2 diabetes was diagnosed in accordance with the criteria of the World Health Organization. Biochemical measurements on admission for renal biopsy were used as baseline characteristics for cross-sectional analysis. Estimated glomerular filtration rate (eGFR) was calculated using a simplified prediction equation proposed by the Japanese Society of Nephrology: eGFR (ml/min/1.73 m^2) = 194× [age (years)]$^{-0.287}$ × [serum creatinine (mg per dl)]$^{-1.094}$ ×0.739 (for females), which is a validated local modification of MDRD [10]. The serum concentrations of creatinine were measured using an enzymatic method.

For immunohistochemistry, 65 Japanese patients who underwent renal biopsy at Department of Medicine and Clinical Science, Kyoto University Hospital between 2000 and 2011 were analyzed. Biopsy-proven diagnoses of all patients during this period are listed in Table S1 in File S1. The subjects examined in this work included DN (n = 19), ORG (n = 10) and non-obese, non-diabetic control subjects who were diagnosed as MGA (n = 19) or MCNS (n = 17). Some cases in these categories were excluded because residual samples available contained less than 10 glomeruli. Definition of DN consisted of (1) more than 5 year duration after the onset of diabetes, (2) existence of micro- or macro-albuminuria, (3) compatible histopathological changes with DN such as glomerular basement membrane thickening, mesangial expansion, nodular sclerosis (Kimmelstiel-Wilson nodules) and/or arteriolar hyalinosis, and (4) exclusion of other causes for renal disorders [11]. ORG was defined morphologically as focal segmental glomerulosclerosis and/or glomerulomegaly in subjects having both obesity and proteinuria, whose definitions were described above [12,13].

For mRNA expression analysis, low quality samples, in which 18S ribosomal RNA (rRNA) levels were lower than the detection sensitivity limit by real-time RT-PCR, were excluded. Subjects enrolled consisted of 22 type 2 diabetic patients who underwent renal biopsy at Osaka City General Hospital between 2000 and 2010, and 6 non-diabetic control subjects, who had biopsy-proven MGA.

Tables 1 and 2 summarize the baseline clinical characteristics of the patients who were examined by immunohistochemical or gene expression analysis, respectively. For light microscopy, the tissue specimens were processed according to standard procedures. Sections were stained with haematoxylin-eosin, periodic acid-Schiff, periodic-acid methenamine silver or Masson trichrome (Fig. S1). The ratios of the number of glomeruli with global sclerosis among that of total glomeruli and the relative areas of tubulointerstitial fibrosis were evaluated independently by two pathologists unaware of diagnosis and clinical data.

Definition of renal outcomes

The following two prognostic indicators were examined by linear regression and logistic regression analyses, respectively: (1) the extent of proteinuria measured at one year post-biopsy, and (2) renal event defined as annual increase in serum creatinine by > 50% from baseline or initiation of chronic dialysis.

Immunohistochemistry

Immunohistochemistry of MRP8 and CD68 was carried out using kidney sections (thickness 4 μm) fixed with 4% buffered paraformaldehyde. After antigen retrieval by citrate buffer, kidney sections were incubated with 10% goat serum, followed by mouse anti-human MRP8 (1:100; BMA biomedicals, Augst, Switzerland) [14] or mouse anti-human CD68 antibodies (1:50; DAKO, Ely, UK), respectively. Primary antibodies were visualized with horseradish peroxidase-conjugated secondary antibody and 3,3-diaminobenzidine tetrahydrochloride (Dako USA, Carpinteria, CA). Nuclei were counterstained with hematoxylin. MRP8-positive cells were counted in more than 10 glomeruli, and MRP8-positive area in tubulointerstitium was measured quantitatively to obtain an average for each subject using MetaMorph 7.5 software (Molecular Devices, Downingtown, PA, USA). Co-localization of CD68- and MRP8-positive cells was evaluated with serial sections. There was neither MRP8 nor CD68 signal in negative controls stained without first antibody (Fig. S2). By preincubation of anti-MRP8 antibody with 20 molar excess of recombinant human MRP8 protein (Life Technologies, Carlsbad, CA, USA) at 4°C overnight, staining was markedly reduced, if not completely, further supporting the specificity of the antibody (Fig. S3).

Evaluation of mRNA expression

Frozen kidney sections were separated into glomeruli and non-glomerulus tissues by laser capture micro-dissection (LM200; Olympus, Tokyo, Japan) as previously described [15]. Total RNA was extracted with RNeasy mini kit (Qiagen, Tokyo, Japan). mRNA expression levels were determined by TaqMan real-time PCR (Applied Biosystems, Foster City, CA, USA) [16,17]. Expression levels of all genes were normalized by 18S rRNA (internal control) levels. See Table S2 in File S1 for primer and probe sequences. Eukaryotic 18S rRNA was detected with Pre-Developed TaqMan Assay Reagents (Applied Biosystems).

Table 1. Baseline clinical characteristics of patients at renal biopsy who were analyzed for MRP8 protein expression by immunohistochemistry.

	Non-obese, non-diabetic control				Between-group differences*
	MGA	MCNS	ORG	DN	
N	19	17	10	19	
Sex (male/female)	10/9	5/12	7/3	14/5	$\chi^2=8.1$, P=0.04
Age (years)	35.5±17.9	36.3±17.4	49.3±16.5	58.3±9.0	P<0.001
Diabetes duration (years)	-	-	-	11.3±6.7	-
BMI (kg/m^2)	19.7±2.1	23.7±3.3	32.0±5.6	24.7±3.5	P<0.001
HbA1c (NGSP, %)	5.6±0.1	5.8±0.2	6.0±0.7	7.1±1.5	P=0.04
Systolic blood pressure (mmHg)	112.2±11.4	113.0±10.2	129.3±10.4	149.4±17.2	P<0.001
Diastolic blood pressure (mmHg)	67.0±6.8	70.2±10.1	82.2±12.7	82.8±10.1	P<0.001
Urinary protein (g/g creatinine)	0.30±0.45	6.83±3.37	1.29±1.24	5.38±4.02	P<0.001
Creatinine (mg/dl)	0.66±0.14	0.68±0.15	0.84±0.23	1.45±0.66	P<0.001
eGFR (ml/min/1.73 m^2)	100.7±24.4	94.0±20.3	75.0±18.9	45.4±19.6	P<0.001
BUN (mg/dl)	13.3±3.7	13.5±6.8	15.6±4.6	23.9±10.3	P<0.001
Total protein (g/dl)	7.0±0.6	4.7±0.8	7.0±0.5	5.8±1.0	P<0.001
Albumin (g/dl)	4.3±0.4	2.2±0.8	4.1±0.6	3.2±0.8	P<0.001
Total cholesterol (mg/dl)	184.7±38.3	442.5±123.2	206.1±27.7	247.3±50.8	P<0.001
Triglyceride (mg/dl)	100.1±84.4	230.8±140.9	124.8±63.7	175.8±75.0	P=0.001
HDL cholesterol (mg/dl)	55.1±82.8	82.8±16.8	53.7±10.8	46.0±10.8	P<0.001
LDL cholesterol (mg/dl)	106.1±30.2	298.2±123.6	127.6±26.0	161.8±47.6	P<0.001
CRP (mg/dl)	0.3±0.9	0.3±0.8	0.3±0.2	0.1±0.2	NS
Global glomerulosclerosis (%)	2.2±4.2	2.5±4.5	22.9±15.9	33.2±17.4	P<0.001
Tubulointerstitial fibrosis (%)	0.6±1.4	0.1±0.5	15.5±9.5	38.6±18.6	P<0.001

MGA: minor glomerular abnormality, MCNS: minimal change nephrotic syndrome, ORG: obesity-related glomerulopathy, DN: diabetic nephropathy, BMI: body mass index, BUN: blood urea nitrogen, CRP: C-reactive protein. Data are means ± SD. *Overall differences between MGA, MCNS, ORG and DN groups were compared by ANOVA.

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MRP8 treatment of macrophages

Bone marrow-derived macrophages were generated from wild-type or TLR4 knockout (KO) mice [18] on C57BL/6J genetic background (Oriental BioService, Kyoto, Japan) as described previously [6]. Briefly, following lysis of red blood cells, bone marrow cells were resuspended in medium containing 20% fetal calf serum and 50 ng/ml recombinant human macrophage colony-stimulating factor (Peprotech, Rocky Hill, NJ, USA), and cultured at 37°C in 5% CO_2 atmosphere. On day 7, macrophages were incubated with recombinant mouse MRP8 (Abnova, Taipei, Taiwan) or vehicle for 4 hours. Polymyxin B (25 μg/ml, Nacalai Tesque, Kyoto, Japan) was added to each well to minimize contamination of endotoxin as described previously [3,19]. No endotoxin was detected at any concentration of MRP8 tested here after incubation with 25 μg/ml of polymyxin B by ToxinSensor Chromogenic LAL Endotoxin Assay Kit (GenScript, Piscataway, NJ, USA). Total RNA from cells was extracted with RNeasy Mini Kit, and mRNA expression levels of interleukin-1 beta (IL-1β), tumor necrosis factor alpha (TNFα) and MRP8 were determined by TaqMan real-time RT-PCR. Expression levels of all genes were normalized by rodent GAPDH levels (internal control, Pre-Developed TaqMan Assay Reagents). Primer and Probe sequences for real-time PCR are listed in Table S2 in File S1.

Statistical analysis

Data are expressed as means ± SD, or means ±95% confidence interval (CI) when appropriate. For the comparison among four groups, one-way or two-way ANOVA with Bonferroni' post-hoc analysis was used, and categorical variables were compared using χ^2 test. Student's unpaired t-test was applied for comparison between two groups as appropriate. Spearman's correlation coefficients were estimated to determine associations between two variables. To examine the effects of baseline covariates determining the extent of glomerular or tubulointerstitial MRP8 expression or urinary protein levels one year after biopsy, univariate and multivariate linear regression analyses were performed. Logistic regression analysis was used to analyze explanatory variables predicting the occurrence of renal event. All data were analyzed using StatView 5.0 software (SAS Institute Inc., Cary, NC, USA). P values <0.05 were considered statistically significant.

Results

We compared MRP8 protein expression levels in the kidneys among DN, ORG and non-obese, non-diabetic control (MGA and MCNS) groups. Immunohistochemical analysis revealed that both glomerular MRP8-positive cell count (Fig. 1A) and tubulointerstitial MRP8-positive area (Fig. 1B) in DN were significantly larger than those in other groups including MGA, MCNS and ORG (P<

Table 2. Baseline clinical characteristics of patients at renal biopsy who were analyzed for MRP8 mRNA expression by real-time RT-PCR.

	MGA	DN	Between-group differences*
N	6	22	
Sex (male/female)	0/6	15/7	$\chi^2 = 8.8$, P = 0.003
Age (years)	38.3±10.4	57.0±11.1	P = 0.001
Diabetes duration (years)	-	14.1±6.8	-
RAS blockade (yes/no)	0/0	18/4	-
BMI (kg/m^2)	19.4±2.0	25.0±3.8	P = 0.002
HbA1c (NGSP, %)	-	7.9±2.0	-
Systolic blood pressure (mmHg)	111.2±13.5	157.9±30.1	P = 0.001
Diastolic blood pressure (mmHg)	67.3±9.0	86.8±11.7	P = 0.001
Urinary protein (g/g creatinine)	0.10±0.11	4.87±4.09	P = 0.009
Creatinine (mg/dl)	0.6±0.2	1.1±0.4	P = 0.004
eGFR (ml/min/1.73 m^2)	92.8±26.6	57.8±27.2	P = 0.009
Total protein (g/dl)	7.0±0.4	6.2±0.9	NS
Albumin (g/dl)	4.2±0.4	3.2±0.7	P = 0.002
Total cholesterol (mg/dl)	213.5±57.2	240.1±71.2	NS
Triglyceride (mg/dl)	172.0±161.4	241.0±137.7	NS
HDL cholesterol (mg/dl)	61.3±12.5	52.5±12.0	NS
LDL cholesterol (mg/dl)	117.8±33.6	137.3±59.1	NS

RAS: renin-angiotensin system. Data are means ± SD. *Differences between MGA and DN groups were compared by unpaired t-test.
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0.01). ORG subjects also showed a tendency of elevated MRP8 expression compared to MGA and MCNS (Fig. 1A, 1B). Furthermore, glomerular MRP8 mRNA expression levels in DN subjects were significantly higher compared to non-DM control subjects (P<0.01, Fig. 1C). In non-glomerulus tissues, MRP8 mRNA expression levels were much lower than those in glomeruli, both in non-DM and DM groups. Abundant MRP8 protein expression in the tubulointerstitium of DN cases was not clearly reflected into increased mRNA expression, which may be partly caused by deposition of blood-derived proteins in the tubulointerstitium as discussed in the next section. As shown in representative photos (Fig. 1D–G, see Fig. S4 in detail), renal biopsy samples from MGA and MCNS subjects showed few MRP8-positive cells in glomeruli (Fig. 1D, 1E and Fig. S4). In ORG subjects, some MRP8-postive cells appeared in glomeruli and tubulointerstitium (Fig. 1F and Fig. S4). In DN subjects, marked increase of MRP8-expressing cells in glomeruli and significant expansion of MRP8-positive areas in the tubulointerstitium were observed in a focal manner (Fig. 1G and Fig. S4). Of note, MRP8-positive cells were absent in nodular sclerosing lesions of diabetic glomeruli (Fig. S4: DN case 2, 3) as described previously for sclerotic lesions in ANCA-associated glomerulonephritis [20]. Paired immunohistochemistry for CD68 and MRP8 in serial sections suggested that MRP8 signals were, at least in part, observed in macrophages expressing CD68 (Fig. 2), as we reported in a mouse model of diabetic nephropathy [6]. Besides, focally injured atrophic tubular epithelial cells also strongly expressed MRP8, which were surrounded by MRP8(+)-, CD68(+)-positive macrophages (Fig. 2, Fig S4: DN case 3-5). In the cases with nephrotic range proteinuria, MRP8 staining was also observed along brush borders of proximal tubules both in MCNS and DN cases (Fig. S4). Since sample number of mRNA expression was too small for multivariate analysis, the following analyses were performed using data from patients studied by immunohistochemistry.

The associations between kidney MRP8 signals and baseline clinical parameters at the time of renal biopsy were analyzed cross-sectionally (Table 3). By univariate analysis, glomerular and/or tubulointerstitial MRP8 protein expression was significantly correlated to age, systolic and diastolic blood pressures, urinary protein, serum levels of creatinine, BUN and HDL cholesterol, eGFR, and extent of global glomerulosclerosis and tubulointerstitial fibrosis. These parameters were further examined by multivariate analysis after excluding diastolic blood pressure, eGFR and BUN because of collinearity. Percentage of tubulointerstitial fibrosis was independently correlated with glomerular MRP8 signals ($\beta = 0.62$, adjusted P = 0.02) and tubulointerstitial MRP8 signals ($\beta = 0.85$, adjusted P<0.001), respectively. Additionally, tubulointerstitial MRP8 signals were also independently correlated with baseline proteinuria ($\beta = 0.20$, adjusted P = 0.01).

Scattered plot analyses between MRP8 signals in glomeruli or tubulointerstitium and clinical parameters indicated that MCNS group had a distinct distribution pattern from other groups, especially as to urinary protein and serum LDL cholesterol levels (Fig. S5A–D, S6A–D). Exclusion of MCNS group improved correlation between MRP8 signals and urinary protein or serum LDL-cholesterol levels (Fig. S5E–F, S6E–F). Therefore, we carried out sub-analysis excluding MCNS patients, and found that urinary protein was an independent factor correlated with glomerular MRP8 signals by multivariate analysis (Table 4; $\beta = 0.36$, adjusted P = 0.03).

Next, we performed linear regression or logistic regression analyses to identify explanatory factors predicting renal outcomes which were extent of proteinuria a year later and renal event within a year. Since there was a good association between glomerular and tubulointerstitial MRP8 signals (Fig. S7; R = 0.67,

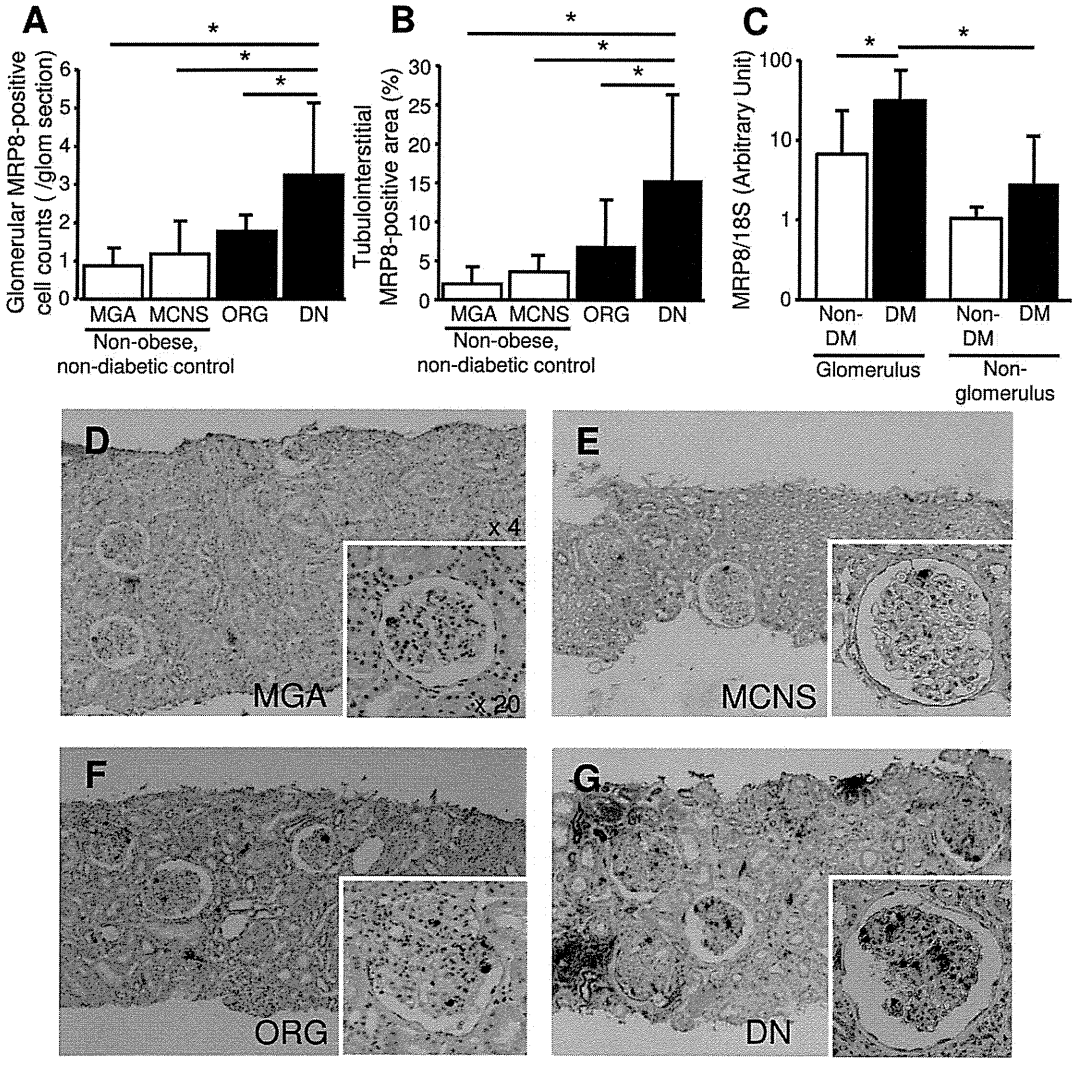

Figure 1. Immunohistochemical and mRNA analyses for MRP8 in kidney biopsy samples. Quantification of glomerular MRP8-positive cell count (A) and tubulointerstitial MRP8-positive area (B). mRNA expression of MRP8 in glomerular and non-glomerular fractions (C). Open bars: non-obese, non-diabetic controls which are MGA or MCNS, closed bars: ORG or DN (A–C). Representative pictures of MGA, MCNS, ORG and DN groups (D–G). MGA: minor glomerular abnormality, MCNS: minimal change nephrotic syndrome, ORG: obesity-related glomerulopathy, DN: diabetic nephropathy. *P<0.01.
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P<0.001), these parameters were alternatively enrolled in further analyses. We evaluated association between baseline parameters and urinary protein at 1 year after renal biopsy by multiple regression analysis. As shown in Table 5, glomerular MRP8 signal ($\beta = 0.59$, adjusted P<0.001) was a predictive factor for the extent of proteinuria a year later, as well as baseline systolic blood pressure ($\beta = 0.21$, adjusted P = 0.04) and baseline proteinuria ($\beta = 0.37$, adjusted P = 0.002) were. These parameters were independent from other known diabetic nephropathy risk factors including renal dysfunction (serum creatinine) and extent of global sclerosis and tubulointerstitial fibrosis [11,21–24]. On the other hand, tubulointerstitial MRP8 signal ($\beta = 0.34$, adjusted P = 0.09) was not an independent predictive factor for urinary protein levels a year later. Renal events occurred in 7 patients (6 in DN and 1 in ORG cases) within a year after renal biopsy. By univariate analysis, not only extent of glomerulosclerosis and tubulointerstitial fibrosis, and glomerular and tubulointerstitial MRP8 signals, but also blood pressures, renal dysfunction and urinary protein levels at baseline were significant predictive factors for the occurrence of renal events. However, by multivariate analysis, there covariates were cancelled out by each other (Table S3 in File S1), likely due to high correlations among these parameters.

Finally, we examined the potency of MRP8 as an endogenous ligand for TLR4 using cultured macrophages. In bone marrow-derived macrophages from wild-type mice, MRP8 protein induced upregulation of proinflammatory cytokine genes such as IL-1β and TNFα and also triggered auto-induction of MRP8, in a dose-dependent manner between 10–1000 ng/ml. These effects of MRP8 were suppressed approximately by two-thirds in macrophages obtained from TLR4 KO mice (P<0.01) (Fig. 3).

Discussion

The present study has demonstrated that MRP8 is abundantly expressed in glomeruli and tubulointerstitium of patients with DN as compared to ORG and non-obese, non-diabetic control (MGA and MCNS). Furthermore, in ORG subjects, MRP8 expression