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Fatty acid binding protein 3 as a potential mediator for diabetic nephropathy in eNOS deficient mouse

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ABSTRACT

In human diabetic nephropathy, glomerular injury was found to comprise lipid droplets, suggesting that abnormal lipid metabolism might take place in the development of diabetic glomerular injury. However, its precise mechanism remains unclear. Fatty acid binding protein (FABP) is currently considered as a key molecule for lipid metabolism. Since diabetic eNOS knockout (KO) mouse is considered to be a good model for human diabetic nephropathy, we here investigated whether FABP could mediate glomerular injury in this model. We found that glomerular injuries were associated with inflammatory processes, such as macrophage infiltration and MCP-1 induction. Microarray assay with isolated glomeruli revealed that among 10 isoforms in FABP family, FABP3 mRNA was most highly expressed in diabetic eNOSKO mice compared to non-diabetic eNOSKO mice. FABP3 protein was found to be located in the mesangial cells. Overexpression of FABP3 resulted in a greater response to palmitate, a satulated FA, to induce MCP-1 in the rat mesangial cells. In turn, the heart, a major organ for FABP3 protein in normal condition, failed to alter its expression level under diabetic condition in either wild type or eNOSKO mice. In conclusion, FABP3 is induced in the mesangial cells and likely a mediator to induce MCP-1 in the diabetic nephropathy.

1. Introduction

A lack of insulin action which stems from either insulin deficiency or insulin resistance causes diabetes. While a major feature of diabetes is the hyperglycemia, an increase in serum fatty acid (FA) could be another feature in diabetes. A lack of insulin function induces lipolysis and then releases the FA into circulation from white adipose tissue. FA is usually a source of energy for several types of cells in case of hypoglycemia whereas an excess amount of FA is likely problematic in our body. In fact, several studies indicate that serum FA could be involved in inflammation, endothelial dysfunction, and insulin resistance [1]. Several mechanisms for the deleterious effects of FA have been proposed. An interesting mechanism could be a perturbation of plasma FA composition which could be an unbalance of satulated FA with unsatulated FA because such unbalance was found to be associated with the metabolic syndrome and low grade inflammation in overweight adolescents [2]. In kidney, free FA bound to albumin could be filtered through glomeruli in vivo, and stimulate the cultured proximal tubular epithelial cells to release MCP-1 [3].

FA serves such roles to maintain physiological cellular function as membrane phospholipid constituents, metabolic substrates, precursors for intracellular signaling molecules and mediators for gene expressions. As such, it is likely that the highly sophisticated mechanism must be required for FA metabolism and such mechanism would be regulated by several factors. In these processes, the fatty acid binding proteins (FABPs) have been postulated to contribute to the cellular FA transports and utilizations [4,5].

Diabetic nephropathy is characterized by unique glomerular injuries, including Kimmelstiel–Wilson nodule, mesangiolysis and glomerular microaneurysm. Interestingly, nodular lesions were found to comprise the lipid droplets [6], suggesting that diabetic glomerular injury might be involved in lipid abnormalities. Recently, we and others have reported that diabetic eNOS knockout (KO) mice featured some types of glomerular injuries resemble

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to those in human diabetic nephropathy [7]. Here, we investigate (1) if FABP is involved in the development of glomerular injury, and (2) if it is the case, what is the function of FABP in the development of glomerular injury in the diabetic eNOSKO mice.

2. Materials and methods

2.1. Experimental protocols

All animal experiments were performed in accordance with the Animal Experimentation Committee of Kyoto University and Tanabe R&D Service Co., Ltd. (Osaka, Japan). Male C57BL/6J-Nos3tm1nc (eNOSKO) mice and C57BL/6J (WT) mice at 7 weeks of age were purchased from Jackson Laboratory (Bar Harbor, ME) or Charles River Laboratories (Wilmington, MA), respectively. Diabetes was induced in both types of mice with intraperitoneal injection of 50 mg/kg/day streptozotocin for five consecutive days. Diabetes was defined as non-fasting blood glucose > 250 mg/dl using a blood glucose meter. Mice were fed a standard laboratory chow ad libitum. Mean blood pressure was measured using CODA Multi-Channel, Computerized, Non-Invasive Blood Pressure System (Kent Scientific Corporation, Torrington, CT) while blood glucose level were determined by GLUCOCARD MyDIA (Arkray, Edina, MN). Urine was collected overnight using metabolic cages. At 20 weeks of age, all mice were sacrificed to obtain blood, kidney and heart.

2.2. Laboratory studies

Urine albumin was measured with Albuwell M (Exocell, Philadelphia, PA). Urinary FABP3 was measured with CircuLex Mouse FABP3/H-FABP ELISA Kit (CY-8055) (CycLex, Nagano, Japan). Serum creatinine, total cholesterol, triglyceride and nonesterified FA were measured by a commercial kit (Oriental Bioservice, Kyoto, Japan).

2.3. Isolation of glomeruli

Mice at 14 weeks of age were anesthetized by isoflurane (Mylan, Washington County, PA) and perfused with 8×10^7 Dynabeads (Life technology, Waltham, MA) diluted in 40 ml of Hank's Balanced Salt Solution through the heart. Kidneys were isolated and digested in collagenase solution (1 mg/ml collagenase A and 100 U/ml deoxyribonuclease I) for 30 min at 37 °C, and then glomeruli containing Dynabeads were gathered by a magnetic particle concentrator. During the procedure, kidney tissues were kept at 4 °C except for the collagenase digestion at 37 °C. Glomeruli were lysed with 350 μL of lysis buffer to extract RNA.

2.4. Microarray analysis

Glomerular RNA was extracted and purified with RNeasy Mini Kit (Qiagen, Chatsworth, CA) before RNA quality was assessed with Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). In accordance with the Agilent Technologies protocol, all samples were processed and a total of 480 ng of probe was hybridized to SurePrint G3 Mouse Gene Expression 8 × 60 K (Agilent Technologies). Fluorescence was detected using G2539A Scanner (Agilent Technologies). The data was analyzed with GeneSpring GX (Agilent Technologies).

2.5. Histological analysis

Formalin-fixed paraffin-embedded sections (2 μm) were stained with the periodic acid-Schiff reagent (PAS) on coronal sections of the kidney for light microscopy. All glomeruli (50–100 glomeruli) were evaluated for mesangial expansion, mesangiolysis and glomerulosclerosis. These abnormalities were evaluated as previously reported [7].

2.6. Immunohistochemistry and immunofluorescence

Formalin-fixed, paraffin-embedded sections were used for immunohistochemistry as previously described [8]. The following antibodies were used as primary antibodies: (1) rabbit anti FABP3 antibody (Abcam, Cambridge, UK), (2) goat anti SMA antibody (Abcam), and (3) goat anti SM22 antibody (LSBio, Seattle, WA). Double immunofluorescence staining was performed for FABP3 with either SMA or SM22. Briefly, the deparaffinization of the sections was incubated with 5% donkey serum for 1 h and then with primary antibodies overnight at 4 °C. After incubation with Alexa Fluor 488-labeled donkey anti rabbit IgG (Invitrogen, Carlsbad, CA) and Alexa Fluor 568-labeled donkey anti goat IgG (Invitrogen) for 1 h at room temperature, sections were mounted with vectashield anti-fade mounting medium (Vector, Burlingame, CA). Single immunofluorescence staining for FABP3 was performed using ImmPRESS REAGENT KIT (Vector). In turn, frozen sections (4 μm) were used for immunofluorescence staining for CD68 with mouse anti-CD68 (Abcam). CD68 infiltration was determined by ratio of CD68 positive area to glomeruli area. Areas were measured using MetaMorph (Molecular Devices, Sunnyvale, CA).

2.7. Quantitative polymerase chain reaction (qPCR)

The first strand cDNA was synthesized from 1 μg of total RNA using ReverTra Ace α (TOYOBO, Osaka, Japan). Several sets of primers were used to detect mRNA expressions. Amount of PCR

Table 1
General characteristics.

Characteristic	WT		eNOSKO	
	NDM ($N = 10$)	DM ($N = 12$)	NDM ($N = 10$)	DM ($N = 13$)
Blood glucose (mg/dl)	114.7 ± 15.6	435.3 ± 118.2[a]	116.4 ± 21.8	569.3 ± 83.3[a,b]
Mean BP (mmHg)	93.7 ± 5.8	99.6 ± 10.4	107.4 ± 9.4[c]	108.5 ± 11.3[d]
Urine albumin (μg/day)	2.6 ± 1.2	18.7 ± 13.7	39.9 ± 31.1	595.1 ± 319.9[a,b,e]
Serum creatinine (mg/dl)	0.09 ± 0.02	0.09 ± 0.02	0.10 ± 0.01	0.14 ± 0.03[a,b]
T.CHO (mg/dl)	111.0 ± 16.0	118.3 ± 7.1	108.7 ± 10.4	161.3 ± 48.5[a,b,e]
TG (mg/dl)	93.2 ± 30.7	100.5 ± 49.4	112.2 ± 50.1	76.0 ± 37.3
NEFA (μEq/L)	865.2 ± 149.5	933.3 ± 193.8	736.1 ± 116.6	921.8 ± 305.4

T.CHO, total cholesterol; TG, triacylglycerol; NEFA, non esterified fatty acids.
[a] $p < 0.001$ vs. NDM WT.
[b] $p < 0.001$ vs. NDM eNOSKO.
[c] $p < 0.05$ vs. NDM WT.
[d] $p < 0.01$ vs. NDM WT.
[e] $p < 0.01$ vs. DM WT.

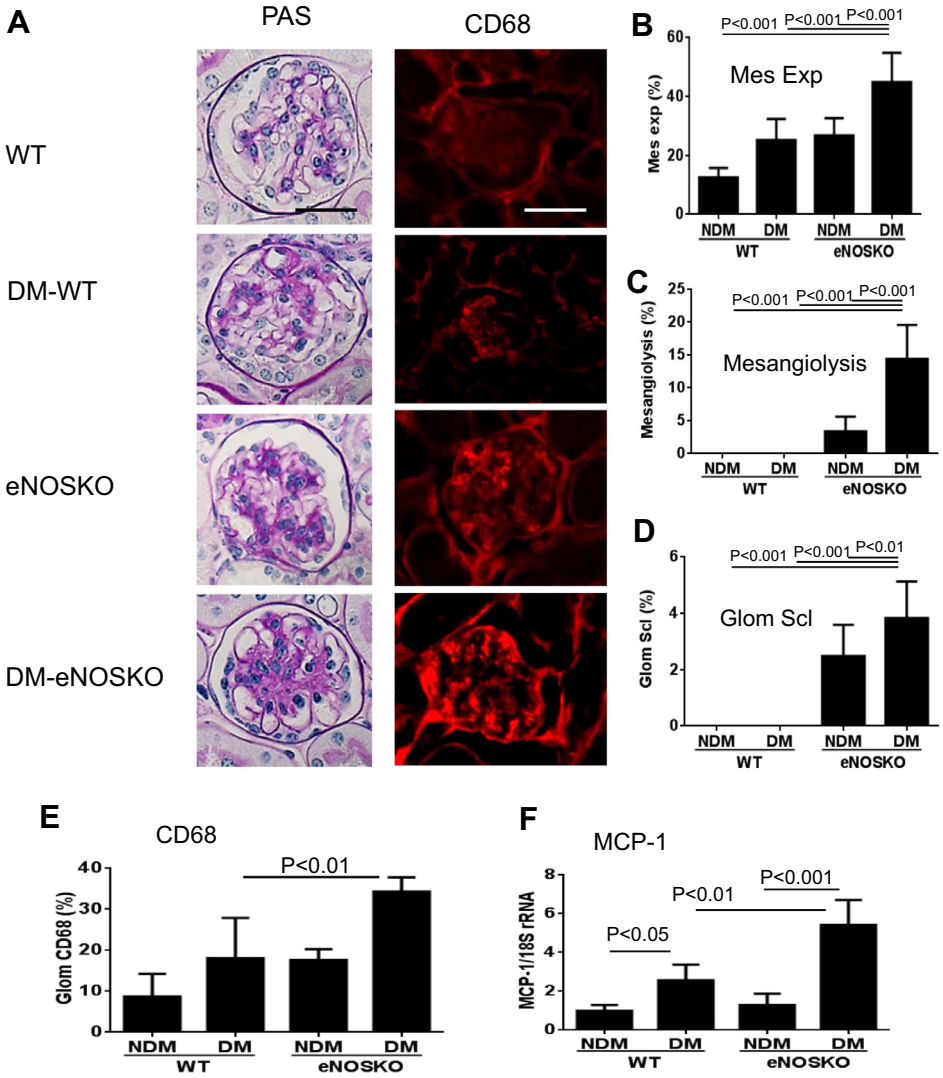

Fig. 1. Glomerular injury is associated with inflammation in diabetic eNOSKO mouse. PAS staining shows that mesangial expansion is prominent in diabetic (DM) eNOSKO mouse compared to WT, DM WT, and eNOSKO mouse (A). Glomerular CD68 signals appear to be increased in DM eNOSKO mouse compared to other mice (A). Quantification of glomerular injury using PAS staining is shown for mesangial expansion (Mes Exp) (B), mesangiolysis (C), and glomerulosclerosis (Glom scl) (D). % positive area for CD68 in glomerulus is shown in (E). MCP1 mRNA expression in isolated glomeruli is shown in (F). NDM, non diabetes; DM, diabetes; Each column consists of mean ± SD. Scale bar, 50 μm.

products was normalized with mouse 18S rRNA mRNA or rat Actin. Following primers were used; 18S rRNA, AGGGGAGAGCGGGTAAGAGA (forward (FW)) and GGACAGGACTAGGCGGAACA (reverse (RV)); mouse MCP1, AGGTCCCTGTCATGCTTCTG (FW) and TCTGGACCCATTCCTTCTTG (RV); mouse FABP3, CTTTGTCGGTACCTGGAAGC (FW) and TGGTCATGCTAGCCACCTG (RV); rat actin, CACCCGCGAGTACAACCTTC (FW) and CCCATACCCACCATCACACC (RV); rat MCP1 ATGCAGTTAATGCCCCACTC (FW) and TTCCTTATTGGGGTCAGCAC (RV).

2.8. Western blotting

Kidney cortex and heart were homogenized and lysed directly with RIPA buffer. Briefly, samples were processed with SDS–PAGE, and electrotransferred onto a PVDF membrane. After overnight incubation with primary antibody at 4 °C, membrane was incubated with secondary antibody for 1 h at room temperature. ECL Prime (GE Healthcare, Buckinghamshire, England) was added to detect bands and figures were shown by Image Quant LAS4000mini (GE Healthcare). FABP3 (Abcam), GAPDH (Millipore) and β actin (SIGMA–ALDRICH, St. Louis, MO) were used as primary antibody. Anti-Mouse HRP (GE Healthcare) and Anti-Rabbit HRP (GE Healthcare) were used as secondary antibody.

2.9. Construction of adenoviral vector

The complementary DNA encoding the full length mouse FABP3 protein was amplified from mouse kidney cDNA. We used the sense primers GGGGACAAGTTTGTACAAAAAAGCAGGCTGCCACCATGGCGGACGCCTTTGTCGGTAC and the antisense GGGGACCACTTTGTACAAGAAAGCTGGGTCTCACGCCTCCTTCTCATAAGTCCG to insert mouse FABP3 gene into donor vector (pDONR221), and induce the recombination reaction for pAd CMV FABP3. The recombinant adenovirus backbone was linearized by digestion with PacI and infected to 293A cells for packing to obtain recombinant protein. The titer was estimated using Adenovirus Hexon Polyclonal HRP conjugate Antibody (Thermo Fisher Scientific, Waltham, MA). An

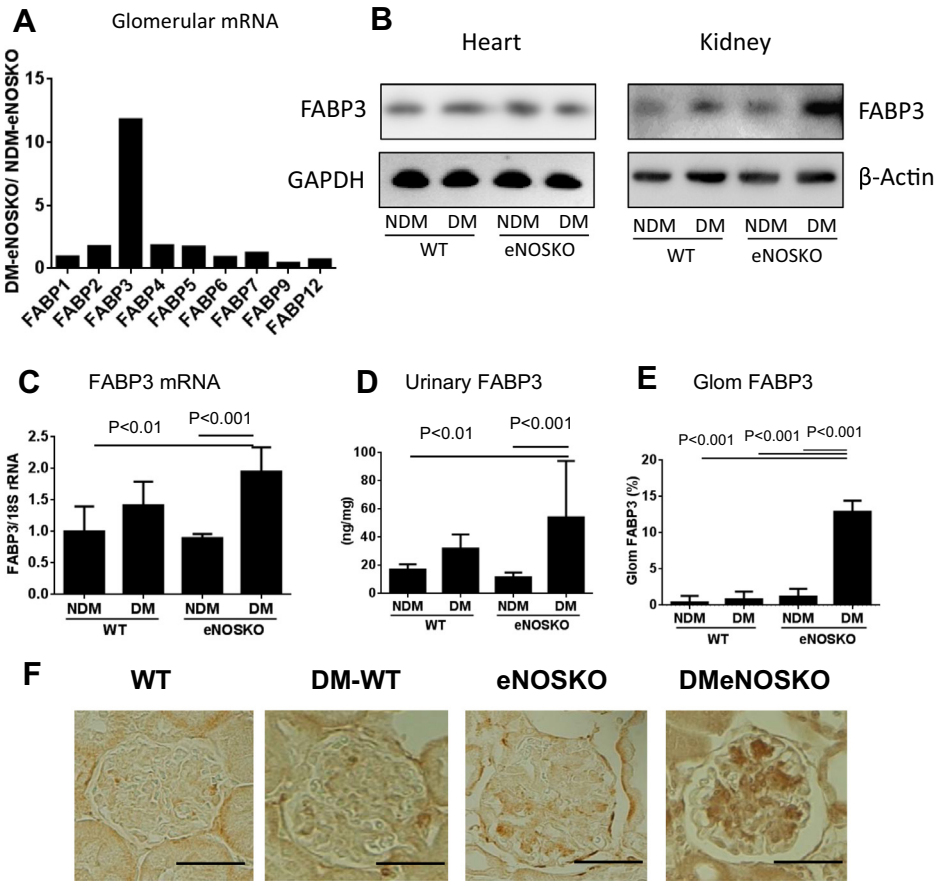

Fig. 2. FABP3 expression in glomerulus of diabetic eNOSKO mice. Microarray analysis demonstrates that FABP3 mRNA is most abundantly expressed in glomeruli of diabetic (DM) eNOSKO mice compared to non diabetic (NDM) eNOSKO mice (A). Western blotting shows that FABP3 protein in the renal cortex is increased in DM-WT mice and appears to be the highest in DM eNOSKO mice (B). In contrast, FABP3 protein expression in the heart shows no change in either diabetes or eNOS deficiency (B). FABP3 mRNA expressions in renal cortex (C), urinary FABP3 protein/creatinine ratio (D), and glomerular FABP3 mRNA expression (E) are shown. Immunohistochemistry demonstrates that FABP3 protein appears to be prominent with the mesangial pattern in glomerulus of DMeNOSKO mice (F). Each column consists of mean ± SD. Scale bar, 50 μm.

adenovirus vector carrying LacZ gene (Ad CMV LacZ) was also used as control.

2.10. FFA assay

Rat mesangial cells (RMC) (ATCC, Manassas, VA) in RPMI1640 with 10% fetal bovine serum (FBS) were infected by adenovirus for 1 h, then medium was replaced with RPMI1640 with 0.5% FBS. After 31 h, palmitate, a satulated FA, was added into medium. Cells were incubated for 16 h and harvested for Western blotting and qPCR. Sodium palmitate (Sigma) was dissolved in 50% ethanol and mixed vigorously with 12.5% FFA-free BSA in PBS.

2.11. Statistical analysis

All values are expressed as mean ± SD. Statistical analysis was performed with ANOVA using Tukey's method to compare four groups. A level of $p < 0.05$ was considered statistically significant.

3. Results

3.1. General characteristics

Streptozotocin induced marked hyperglycemia in both wild type (WT) and eNOSKO mice. A lack of eNOS was associated with higher blood pressure whereas such tendency was maintained after STZ injection. Renal function was reduced as serum creatinine as well as urinary albumin excretion was significantly elevated in diabetic eNOSKO mice compared to other groups. In terms of lipid profile, serum cholesterol was also higher in diabetic eNOSKO mice than that in other mice while serum triglyceride did not show any significant difference between groups. Diabetic condition tended to increase non esterified FA (NEFA) in both WT and eNOSKO mice while it did not reach significance (Table 1).

3.2. Glomerular histology

As reported previously [7], diabetic wild type mice exhibited mesangial expansion while severer glomerular injuries, such as mesangiolysis and glomerulosclerosis were induced under diabetic condition in eNOSKO mice (Fig. 1A–D). With respect to inflammation, CD68, a macrophage marker, and MCP-1 were also higher in glomeruli of diabetic eNOSKO mice compared to wild type mice (Fig. 1E and F).

3.3. Involvement in FABP3 in diabetic nephropathy

Microarray analysis using glomerular mRNA showed that among 10 isoforms, FABP3 mRNA expression was most abundantly expressed in diabetic eNOSKO compared with non diabetic eNOSKO mice (Fig. 2A). FABP3 protein in the renal cortex appeared to be induced under diabetic condition while the expression level appeared to be the highest in diabetic eNOSKO mice compared to mice in other groups (Fig. 2B). Such pattern was mirrored to that

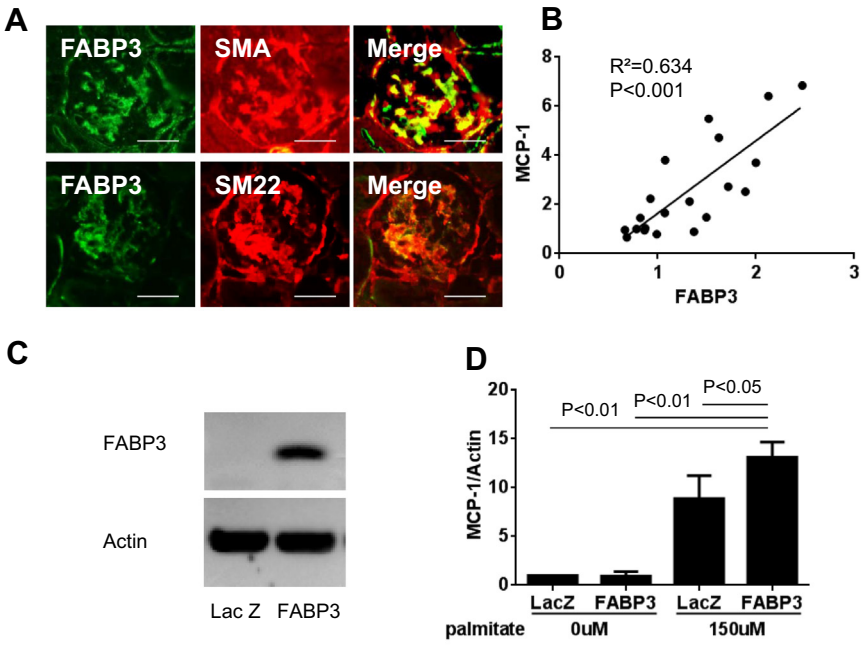

Fig. 3. Mesangial FABP3 causes inflammation. Immunofluorescence demonstrates that FABP3 is overlapped with α-smooth muscle actin (SMA) and SM22 (A). In mRNA expression, association of MCP-1 with FABP3 is found to be positive in this study (B). Rat mesangial cells with Adenovirus coding FABP3 show FABP3 protein expression in contrast to cells with adenovirus coding LacZ, as a control (C). MCP-1 mRNA expression in response to 150 μM palmitate in rat mesangial cells is shown (D). Each column consists of mean ± SD. Scale bar, 50 μm.

for mRNA in the renal cortex (Fig. 2C) as well as urinary FABP3 level (Fig. 2D). An increase in glomerular mRNA induction was likely more specific in diabetic eNOSKO mice compared to other groups (Fig. 2E), and such tendency was also confirmed by immunohistochemistry. In addition, FABP3 protein likely showed the mesangial pattern (Fig. 2F). In turn, FABP3 is known to be dominantly expressed in the heart, and therefore we examined the level of FABP3 in the heart of this animal model. However, the heart did not show any response to diabetic condition in either wild type or eNOSKO mice (Fig. 2B).

3.4. Mesangial FABP3 for MCP-1 expression

FABP3 was predominantly overlapped with smooth muscle actin and sm22, both of which are markers for injured mesangial cells [9]. Since free FA was reported to induce MCP-1 expression [3], we examine the association of FABP3 with MCP-1 expression in isolated glomeruli from diabetic eNOSKO mice. As shown in Fig. 3B, we found a positive association between FABP3 mRNA and MCP-1 mRNA in the isolated glomeruli of this model. In the cultured rat mesangial cells, we induced FABP3 protein using adenovirus system to examine the role of FABP3 (Fig. 3C). We found that an induction of MCP-1 in response to palmitate was significantly enhanced by an overexpression of FABP3 (Fig. 3D). It is interesting to note that angiogensin II (10^{-8}–10^{-6}M), high glucose (30 mM), TGF-β (0.2–20 ng/ml), and palmitate (30–150 μM) failed to induce FABP3 mRNA in the rat mesangial cells (data not shown).

4. Discussion

An increase in serum FA accompanied with hyperglycemia could be another risk factor for the development of diabetic nephropathy. FA is generally metabolized by sophisticated mechanisms in which FABPs play a key role in its metabolism. Currently 10 FABP isoforms have been identified, and we found that FABP3 was most dominantly and significantly induced in glomeruli of diabetic eNOSKO mice compared to non-diabetic eNOSKO mice. Interestingly, it was found that mesangial cell was capable of inducing FABP3 in the glomerulus of this mouse model. The up-regulated FABP3 likely contributed to develop glomerular inflammation as overexpression of FABP3 in the mesangial cells was proved to enhance the MCP-1 induction in response to free FA in the cultured mesangial cells. Consistently, FABP3 expression was associated with MCP-1 expression and CD68 cells infiltration in glomerular injury of this animal model.

Human diabetic nephropathy is characterized by several types of glomerular injuries, including nodular sclerosis, mesangiolysis and capillary microaneurysm. However, precise mechanisms for such injuries remain unknown. Interestingly, electronic microscopy demonstrated that nodular lesions were composed of multiple factors, including lipid droplet [6]. Likewise, both Akita mouse and db/db mouse were found to develop lipid depositions in the glomerulus as the disease progressed [10,11]. A mechanism for the lipid deposition was due in part to an increase in FA synthesis in the diabetic kidney [10,11]. These data suggest that an increase of FA levels in serum and kidney could mediate the development of diabetic glomerular injury. However, it remains unclear as to how FA mediates the pathophysiological process in the diabetic nephropathy.

Implication of FA in the inflammatory response has been studied by several investigators. For instance, an administration of palmitate, a satulated FA, was found to be enhance diacylglycerol levels in skeletal muscle cells, leading to activation of protein kinase C/NFκB pathway [12]. Likewise, the proximal tubular epithelial cells showed a response to palmitate to induce MCP-1 [3]. Consistently, a clinical study found the positive association of plasma FA with low grade inflammation in overweight adolescents [2]. In this study, we found an induction of FABP3 in glomeruli, in particular the injured mesangial cells of diabetic eNOSKO mice. FA-induced MCP-1 induction was also enhanced by an overexpression of FABP3 in mesangial cells. Consistently, an accumulation of CD68, a marker of macrophage, and an

induction in MCP-1 expression were also associated with FABP3 expression. Perhaps, the mesangial FABP3 could cause inflammation in diabetic condition.

FABPs are small, highly-expressed proteins that reversibly bind to FA and other lipophilic molecules. FABP which locates on plasma membrane are able to facilitate FA uptake into cells while intracellular FABPs can transport FA to other locations, including nuclear receptors. Distinct mechanisms could be operated in different organs and cells as 10 isoforms of FABPs are distinctly distributed in various tissues in mammals [4]. Among them, FABP3 is most predominantly expressed in the heart and less in the skeletal muscle, kidney, stomach, and testis [4]. In human kidney, there are two types of FABPs; one is FABP3 and the other is FABP1 [13]. FABP1 is expressed in the proximal tubular epithelial cells whereas FABP3 was found to be present in the distal tubular epithelial cells [13]. Nonetheless, the fact that FABPs are expressed in normal condition suggests that such protein could be indispensable for maintaining physiological condition in several types of cells or organs.

Recently, Nauta et al. have reported that there was a significant increase in urinary FABP3 concentration in diabetic patients, and importantly such elevation in the urine was detected prior to the onset of microalbuminuria [14]. Given the FABP3 expression in distal tubular epithelial cells, such elevation in urinary FABP3 would be accounted for by the distal tubular damage. Here we showed that the mesangial cells expressed FABP3, indicating that mesangial cells could be another source of urinary FABP3 in diabetic patients. As such, high in urinary FABP3 concentration could indicate the distal tubular damages as well as the glomerular injury in diabetic patients.

In conclusion, we found that FABP3 was induced in advanced diabetic nephropathy in eNOSKO mice. Mesangial expression of FABP3 could contribute to the induction of inflammatory response in the process of diabetic glomerular injury in the mouse.

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