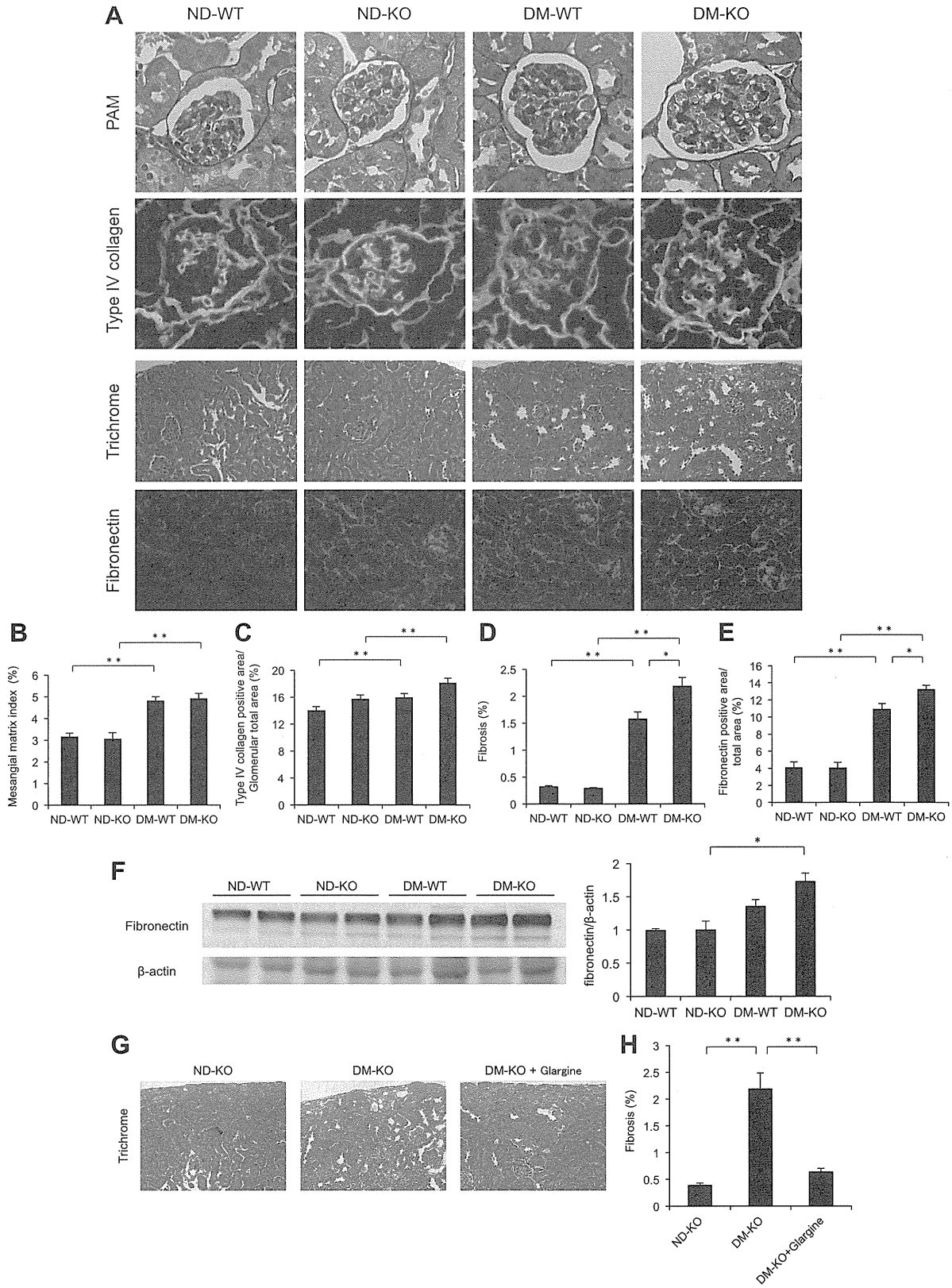
Quantitative analysis of renal cortex gene expression. RNA was isolated from the renal cortex 12 wk after treatment using an RNeasy Mini kit (Qiagen, Valencia, CA). Single-strand cDNA was synthesized from the extracted RNA using an RT-PCR kit (PerkinElmer, Foster City, CA). Quantitative RT-PCR (qRT-PCR) was performed to analyze the mRNA expression of CD14, monocyte chemoattractant protein (MCP)-1, transforming growth factor (TGF)-β, and osteopontin (OPN) in the renal cortex, using StepOnePlus (Applied Biosys-



tems, Tokyo, Japan) and FastStart SYBR Premix Ex Taq II (Takara Bio, Otsu, Japan). The primers were purchased from Takara Bio. The results of qRT-PCR were evaluated by the comparative Ct method using GAPDH as the invariant control gene.

Mitochondrial ROS detection. Mitochondrial ROS were detected using MitoTracker Red CM-H_2XRos (Molecular Probes, Eugene, OR) and MitoTracker Green FM (Molecular Probes). Briefly, renal sections were incubated with 10 μM MitoTracker Red CM-H_2XRos and MitoTracker Green FM at room temperature for 1 h. Unbound dye was then removed, and fluorescence was analyzed using a fluorescence microscope (BX51; Olympus, Tokyo, Japan).

Cell culture and treatment. Murine proximal tubular epithelial (mProx24) cells were generously provided by Dr. Takeshi Sugaya (CMIC) and cultured as reported previously (21). mProx24 cells were cultured in DMEM supplemented with 5.5 mM D-glucose (low glucose), 10% FBS, 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM L-glutamine. siRNA experiments were performed using MT siRNA (sc-35926; Santa Cruz Biotechnology) and scrambled siRNA (sc-37007; Santa Cruz Biotechnology). mProx24 cells were transfected with 12.5 nM MT siRNA or scrambled siRNA in the presence of Lipofectamine RNAiMAX (Invitrogen). After siRNA transfection for 24 h, the cells were stimulated with 25 mM D-glucose (high glucose) for 24 h. Individual experiments were repeated at least three times with different lots or preparation of cells.

Quantitative analysis of gene expression in mProx24 cells. Total RNA was prepared from cells using an RNeasy Mini kit (Qiagen) as described above. Nox4, MCP-1, TGF-β, and OPN mRNA expression levels in mProx24 cells were measured using qRT-PCR, as described above.

ELISA. Levels of MT-1/-2 in kidney tissue and mProx24 cells were measured by an ELISA system (Frontier Science, Ishikari, Japan) according to the manufacturer's protocol.

Statistical analysis. All values are given as means ± SE. Statistically significant differences between groups were examined using one-way ANOVA followed by Scheffé's test. A P value <0.05 was considered statistically significant.

RESULTS

STZ induced greater renal damage in $MT^{-/-}$ mice compared with $MT^{+/+}$ mice. The metabolic data are summarized in Table 1. The UAE was significantly higher in diabetic mice than in nondiabetic mice. Moreover, UAE was significantly increased in diabetic $MT^{-/-}$ mice compared with diabetic $MT^{+/+}$ mice at 12 wk after STZ injection (120.66 ± 18.66 vs. 92.92 ± 14.76 μg/day; $p < 0.05$). Glycated hemoglobin and relative kidney weight were increased and body weight was decreased in diabetic mice compared with nondiabetic mice. There were no significant differences in glycated hemoglobin, relative kidney weight, and body weight between diabetic $MT^{+/+}$ mice and diabetic $MT^{-/-}$ mice.

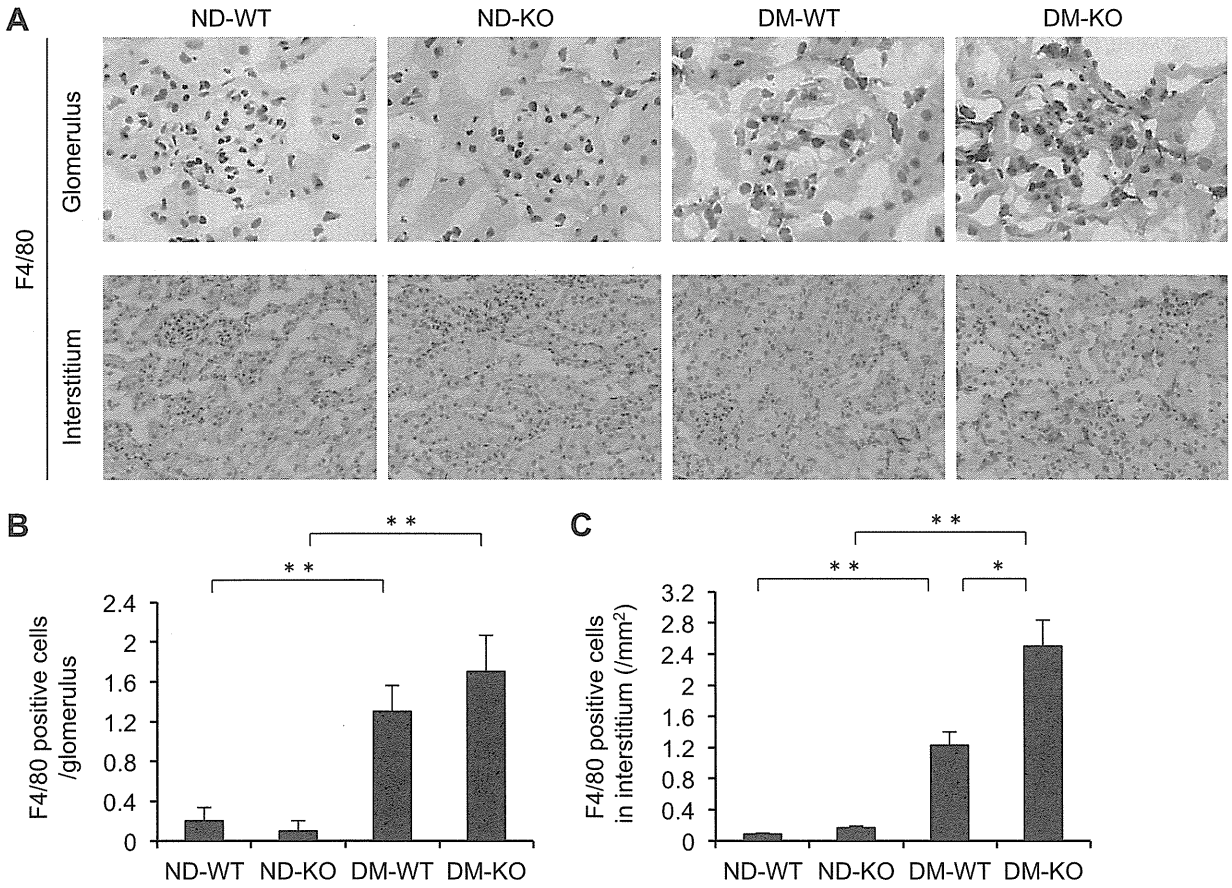


Fig. 2. MT deficiency promotes macrophage infiltration into the interstitium in diabetic mice. *A*: macrophage infiltration into the glomeruli and the interstitium was clearly evident in diabetic mice and was significantly increased in DM-KO compared with DM-WT in the interstitium. Original magnifications: ×400 for glomeruli and ×100 for interstitium. *B*: number of intraglomerular macrophages. Values are means ± SE. $^{**}P < 0.01$. *C*: number of macrophages in the interstitium. Values are means ± SE. $^{*}P < 0.05$. $^{**}P < 0.01$.

STZ induced more severe interstitial fibrosis in $MT^{-/-}$ mice compared with $MT^{+/+}$ mice. Kidneys were isolated and processed for pathological analysis using PAM staining, Masson's trichrome staining, and immunofluorescence for type IV collagen and fibronectin (Fig. 1*A*). As revealed by PAM and type IV collagen staining, glomerular hypertrophy and mesangial matrix expansion were clearly observed in both $MT^{+/+}$ and $MT^{-/-}$ mice after STZ injection. However, morphometric analysis of PAM and the type IV collagen-positive area showed no significant differences between $MT^{-/-}$ and $MT^{+/+}$ mice (Fig. 1, *B* and *C*). Masson's trichrome staining showed significantly increased interstitial fibrosis in diabetic $MT^{+/+}$ mice, which was further increased in diabetic $MT^{-/-}$ mice (1.58 ± 0.13 vs. $2.18 \pm 0.16\%$; $P < 0.05$) (Fig. 1*D*). Immunofluorescent staining and Western blotting for fibronectin also showed the same tendency (Fig. 1, *E* and *F*). Furthermore, interstitial fibrosis in diabetic $MT^{-/-}$ mice was attenuated by insulin treatment, suggesting that interstitial fibrosis observed in STZ-induced diabetic mice was mediated by hyperglycemia (Fig. 1, *G* and *H*). Collectively, these results demonstrate that MT deficiency accelerates interstitial fibrosis in STZ-induced diabetes.

Macrophage infiltration into the interstitium was increased in diabetic $MT^{-/-}$ mice compared with diabetic $MT^{+/+}$ mice. The numbers of macrophages in both the glomeruli and interstitium were remarkably higher in diabetic mice compared with nondiabetic mice (Fig. 2*A*). There was no difference in macrophage infiltration into the glomeruli in response to STZ treatment between $MT^{-/-}$ and $MT^{+/+}$ mice (Fig. 2*B*). However, macrophage infiltration into the interstitium was increased in diabetic $MT^{-/-}$ mice compared with diabetic $MT^{+/+}$ mice (2.50 ± 0.34 vs. 1.23 ± 0.18; $P < 0.01$) (Fig. 2*C*). These findings indicate that MT deficiency accelerates macrophage infiltration into the interstitium of the diabetic kidney.

MT-1/-2 expression was increased in diabetic $MT^{+/+}$ mice. We determined the levels of MT-1/-2 in kidney tissue by ELISA (Fig. 3*A*). The MT-1/-2 level in renal tissue was strongly upregulated in diabetic $MT^{+/+}$ mice compared with control $MT^{+/+}$ mice. In contrast, MT-1/-2 was hardly detected in the kidneys of control and diabetic $MT^{-/-}$ mice. To localize MT-1/-2 in the diabetic $MT^{+/+}$ kidney, we performed double immunofluorescence for MT-1/-2, and AQP1 (a marker of proximal tubular epithelial cells) or THP (a marker of distal tubular epithelial cells). As shown in Fig. 3*B*, MT-1/-2 was predominantly localized in the proximal tubular epithelial cells, and to a lesser extent in the distal tubular epithelial cells of the diabetic kidney.

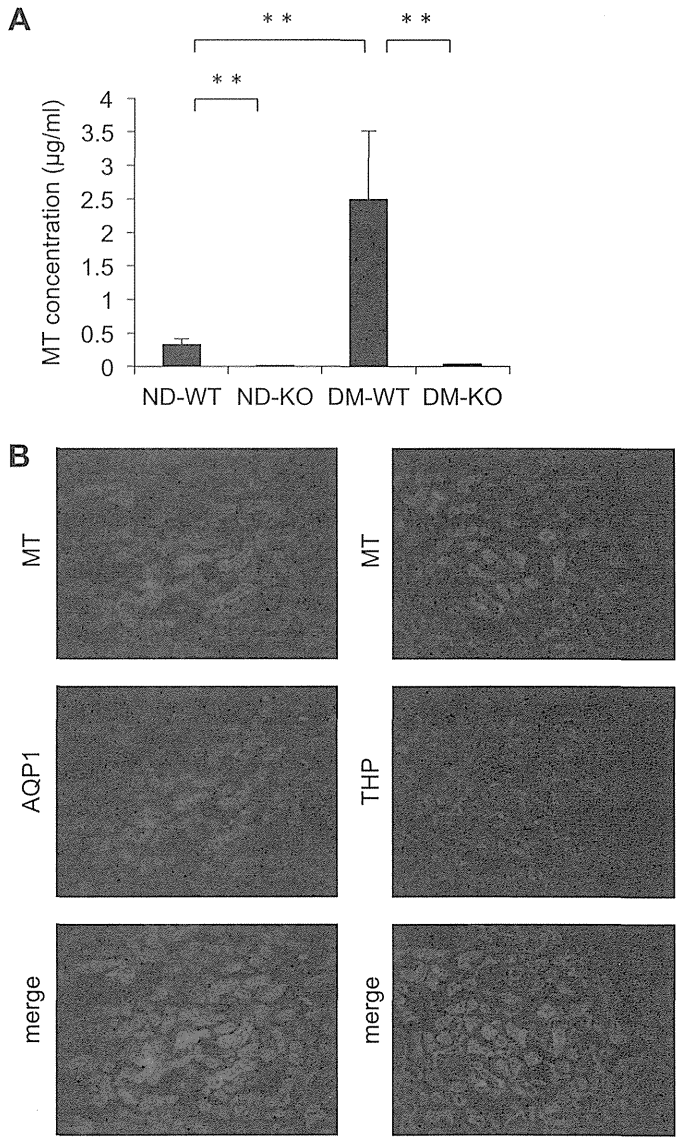


Fig. 3. MT-1/-2 expression is upregulated in the diabetic kidney. *A*: ELISA was performed as described in MATERIALS AND METHODS. Twelve weeks after induction of diabetes, MT-1/-2 was predominantly expressed in the renal cortex of DM-WT compared with ND-WT. In contrast, MT-1/-2 was hardly detected in the kidneys of ND-KO and DM-KO. Values are means ± SE. $^{**}P < 0.01$. *B*: representative photomicrographs of double immunofluorescent staining. MT-1/-2 expression was predominantly localized in the proximal tubular epithelial cells, and to a lesser extent in the distal tubular epithelial cells of the diabetic kidneys. Original magnifications: ×100.

Macrophage and inflammatory gene expression in the renal cortex were higher in diabetic $MT^{-/-}$ mice compared with diabetic $MT^{+/+}$ mice. qRT-PCR analyses of kidney tissues demonstrated increased gene expression of the macrophage marker CD14 in the diabetic groups, and expression of this gene was increased in diabetic $MT^{-/-}$ mice compared with diabetic $MT^{+/+}$ mice (Fig. 4*A*). Similarly, diabetes induction increased the renal expression of several proinflammatory and proatherogenic genes, including MCP-1, TGF-β, and OPN (Fig. 4, *B–D*). Notably, MCP-1 is a key chemokine involved in macrophage recruitment that plays a significant role in diabetic nephropathy, and the absence of MCP-1 significantly reduces diabetic renal injury. Similarly, TGF-β and OPN are also critical inflammatory cytokines involved in diabetic nephropathy. To investigate the involvement of NF-κB in the induction of inflammatory cytokines, we performed Western blotting. The expression of NF-κB was increased in diabetic $MT^{-/-}$ mice compared with diabetic $MT^{+/+}$ mice (Fig. 4*E*). Collectively, these data indicate that MT deficiency accelerates diabetes-induced macrophage recruitment and inflammatory gene expression in the kidney.

Renal ROS generation was increased in diabetic $MT^{-/-}$ mice compared with diabetic $MT^{+/+}$ mice. To evaluate oxidative stress in the kidney, renal sections were immunostained for 4-HNE. This revealed that ROS were generated predominantly in tubular epithelial cells of diabetic $MT^{+/+}$ and $MT^{-/-}$ mice,

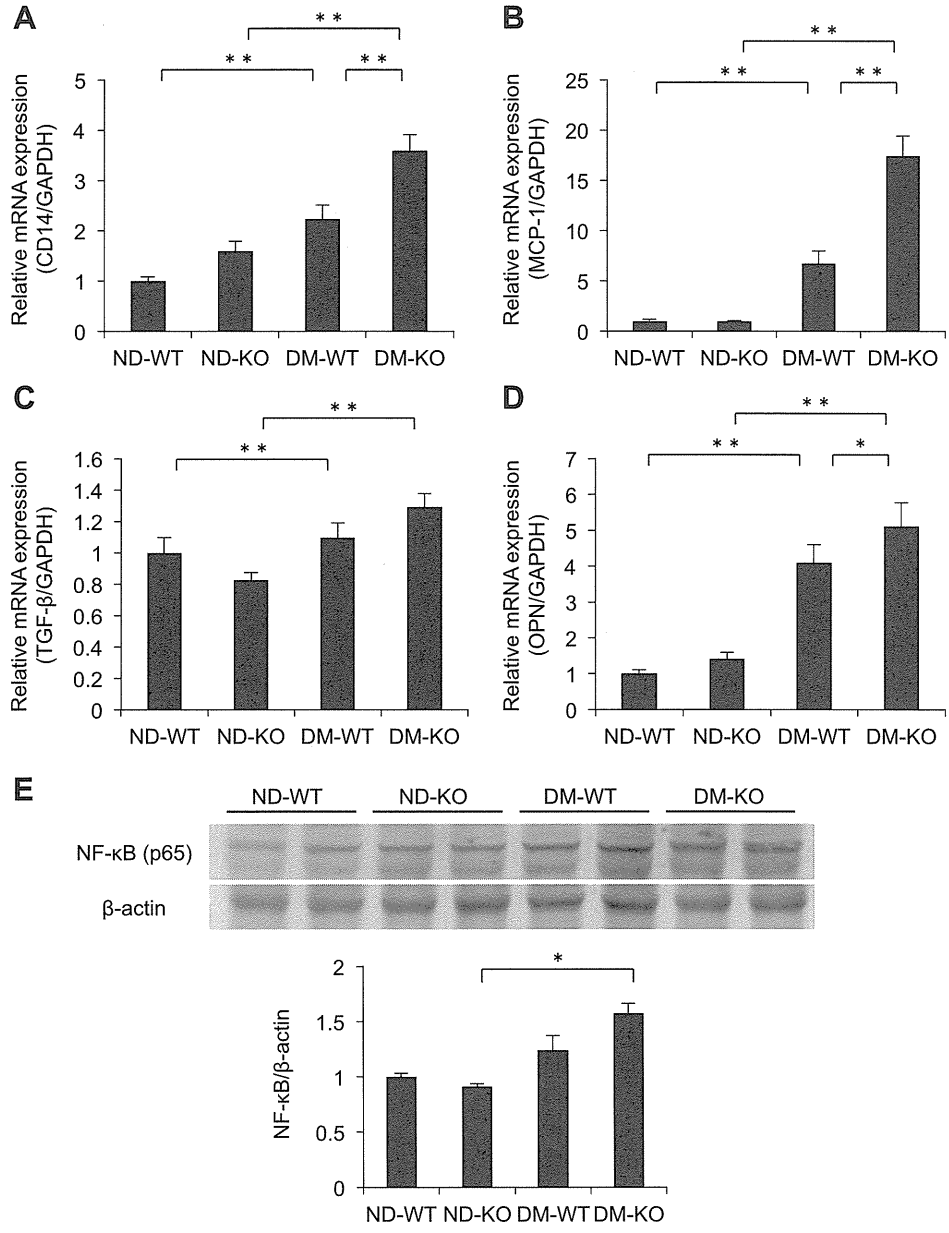


Fig. 4. MT deficiency accelerates diabetes-induced renal inflammation and macrophage infiltration. *A–D*: quantitative RT-PCR analysis of the expression levels of the macrophage marker CD14 and monocyte chemoattractant protein (MCP)-1 showed that MT deficiency promoted diabetes-induced macrophage infiltration into the kidney. Transforming growth factor (TGF)-β and osteopontin (OPN) mRNA levels were increased in the kidney in diabetic mice compared with nondiabetic mice, and were similar between DM-WT and DM-KO. mRNA levels were normalized to GAPDH. Values are means ± SE. $*P < 0.05$. $**P < 0.01$. *E*: Western blot analysis of NF-κB protein expression. NF-κB (p65) is significantly upregulated in DM-KO compared with ND-KO. Quantification was performed by densitometry of 3 independently performed experiments with normalization by β-actin. Values are means ± SE. $*P < 0.05$.

and to a lesser extent in the interstitium of nondiabetic $MT^{+/+}$ and $MT^{-/-}$ mice (Fig. 5*A*). Moreover, we assessed mitochondrial ROS production using MitoTracker Red CM-H_2XRos and MitoTracker Green FM staining. The intensity of MitoTracker Red CM-H_2XRos was higher in diabetic $MT^{-/-}$ mice compared with diabetic $MT^{+/+}$ mice (Fig. 5*B*). These findings suggest that MT deficiency increases diabetes-induced mitochondrial ROS in the interstitium of the kidney.

Mitochondrial morphology deteriorated in diabetic $MT^{-/-}$ mice. To confirm the beneficial effects of MT on generating mitochondrial ROS, we examined renal morphology in more detail using electron microscopy. The number of swollen mitochondria was increased, and cristae were also prominently reduced in renal proximal tubular cells of diabetic $MT^{+/+}$ mice compared with nondiabetic $MT^{+/+}$ mice, and were further increased in diabetic $MT^{-/-}$ mice compared with diabetic $MT^{+/+}$ mice (Fig. 6). These results indicate that MT deficiency impaired mitochondrial function in renal proximal tubular epithelial cells in the diabetic kidney.

ROS and inflammatory gene expression levels were increased by knockdown of MT in cultured proximal tubular epithelial cells. Mouse mProx24 renal proximal tubular epithelial cells were transfected with MT siRNA or scrambled siRNA as a control and subjected to qRT-PCR analyses and ELISA. MT mRNA and protein expression was significantly inhibited in MT knockdown cells compared with control cells (Fig. 7, *A* and *B*). High-glucose-induced Nox4 mRNA expression was increased in MT knockdown cells (Fig. 7*C*). To evaluate mitochondrial ROS in the mProx24 cells, we performed double staining using MitoTracker Red CM-H_2XRos and MitoTracker Green FM. The intensity of MitoTracker Red CM-H_2XRos was increased in mProx24 cells transfected with MT siRNA (Fig. 7*G*). Similarly, expression levels of inflammatory genes including MCP-1, TGF-β, and OPN were upregulated by MT RNAi (Fig.

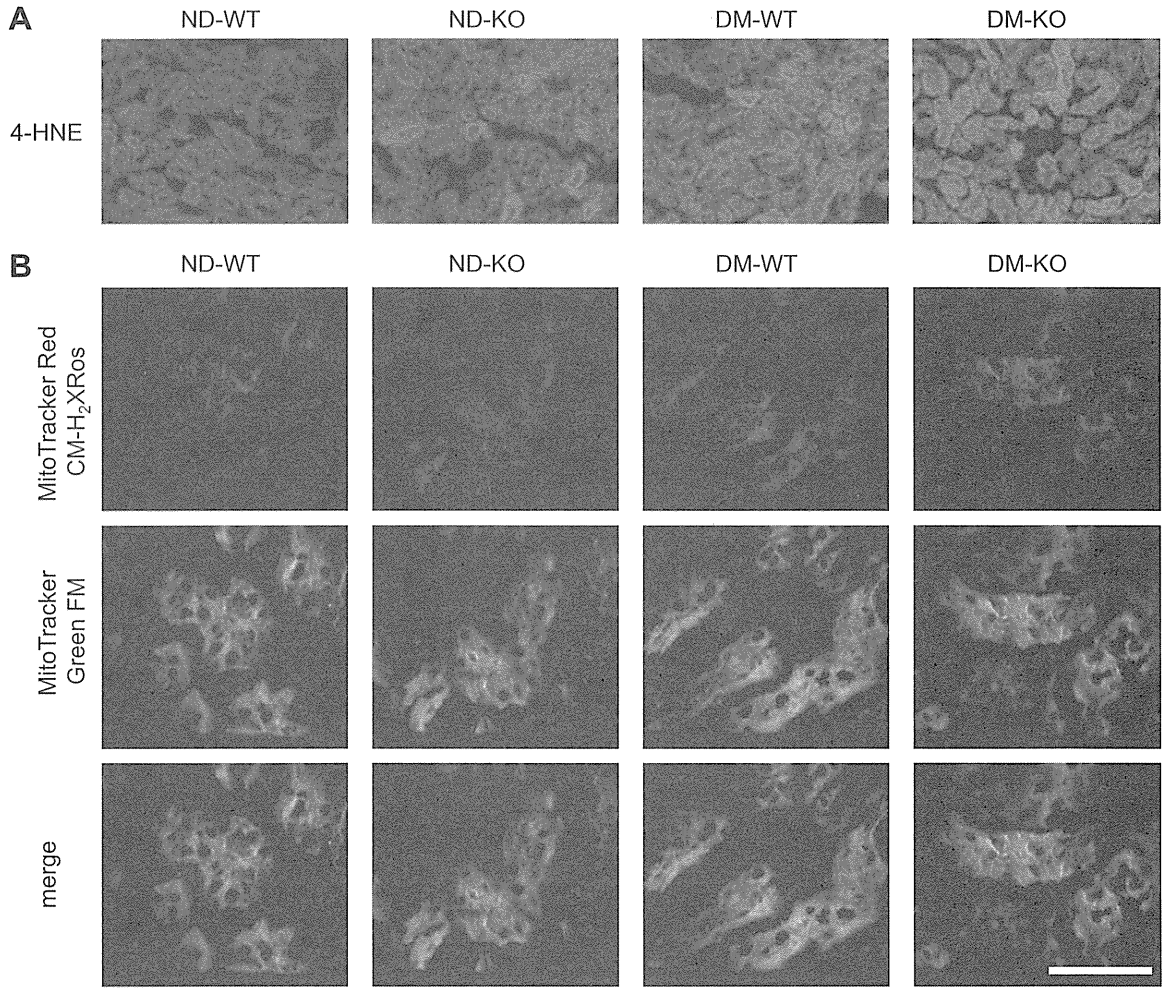


Fig. 5. MT deficiency induced accumulation of mitochondrial reactive oxygen species (ROS) in the diabetic kidney. *A*: oxidative stress was evaluated by fluorescence microscopy examinations using anti-4-hydroxynonenal (4-HNE) antibody. The intensity of 4-HNE was evident in DM-KO. Original magnification: ×100. *B*: mitochondrial ROS production was detected by fluorescence microscopy examinations using MitoTracker Red CM-H_2XRos and MitoTracker Green FM. The intensity of MitoTracker Red CM-H_2XRos was significantly increased in DM-KO compared with DM-WT. Original magnification: ×100.

7, *D–F*). These findings suggest that knockdown of MT exacerbates high-glucose-induced oxidative stress and inflammation in renal proximal tubular epithelial cells.

DISCUSSION

In the present study, we demonstrated that MT deficiency accelerated albuminuria and interstitial fibrosis without affecting blood glucose levels in STZ-induced diabetic mice. Macrophage infiltration in the interstitium of the diabetic kidney and the expression of inflammatory genes, including MCP-1, TGF-β, and OPN, were increased in diabetic $MT^{-/-}$ mice. Furthermore, mitochondrial ROS were increased and mitochondria were fragmented in diabetic $MT^{-/-}$ mice. In vitro studies with proximal tubular epithelial cells revealed that knockdown of MT increased the expression of Nox4, which was associated with oxidative stress, and expression of inflammatory genes. Our findings suggest that MT has antioxidative and anti-inflammatory effects in diabetic kidneys and prevents the development of diabetic nephropathy, independently of blood glucose levels.

MT comprise a family of low-molecular-weight, cysteine-rich, ubiquitous, and inducible intracellular proteins that bind to heavy metals such as zinc, copper, and cadmium and participate in metal homeostasis and detoxification (1). The mammalian MT family comprises four isoforms: MT-1, MT-2, MT-3, and MT-4. MT-3 is predominantly brain specific and is expressed in neurons and stimulated glial cells (16), while the two major isoforms, MT-1/-2, are expressed in most organs. However, the expression of MT in the kidney has been unclear. This and our recent study demonstrated that MT-1/-2 were highly induced in renal proximal tubular epithelial cells in diabetic mice (Fig. 3) and rats (21). This study also showed that MT has previously been reported to be a potent antioxidant; protecting cells from oxidative damage (2, 5, 9, 17, 18). We therefore hypothesized that MT may be induced in the kidney as an antioxidative protein and thus protect the kidney from diabetes-induced ROS and inflammation.

Many studies have proposed an important role of oxidative stress in the pathogenesis of diabetic nephropathy (3, 11, 29). We evaluated oxidative stress in the kidney by assessing mitochondrial ROS generation. MitoTracker Red CM-H_2XRos and MitoTracker Green FM staining revealed that mitochondrial ROS were increased in the interstitium, mainly in the tubular epithelial cells, of diabetic $MT^{-/-}$ mice compared with

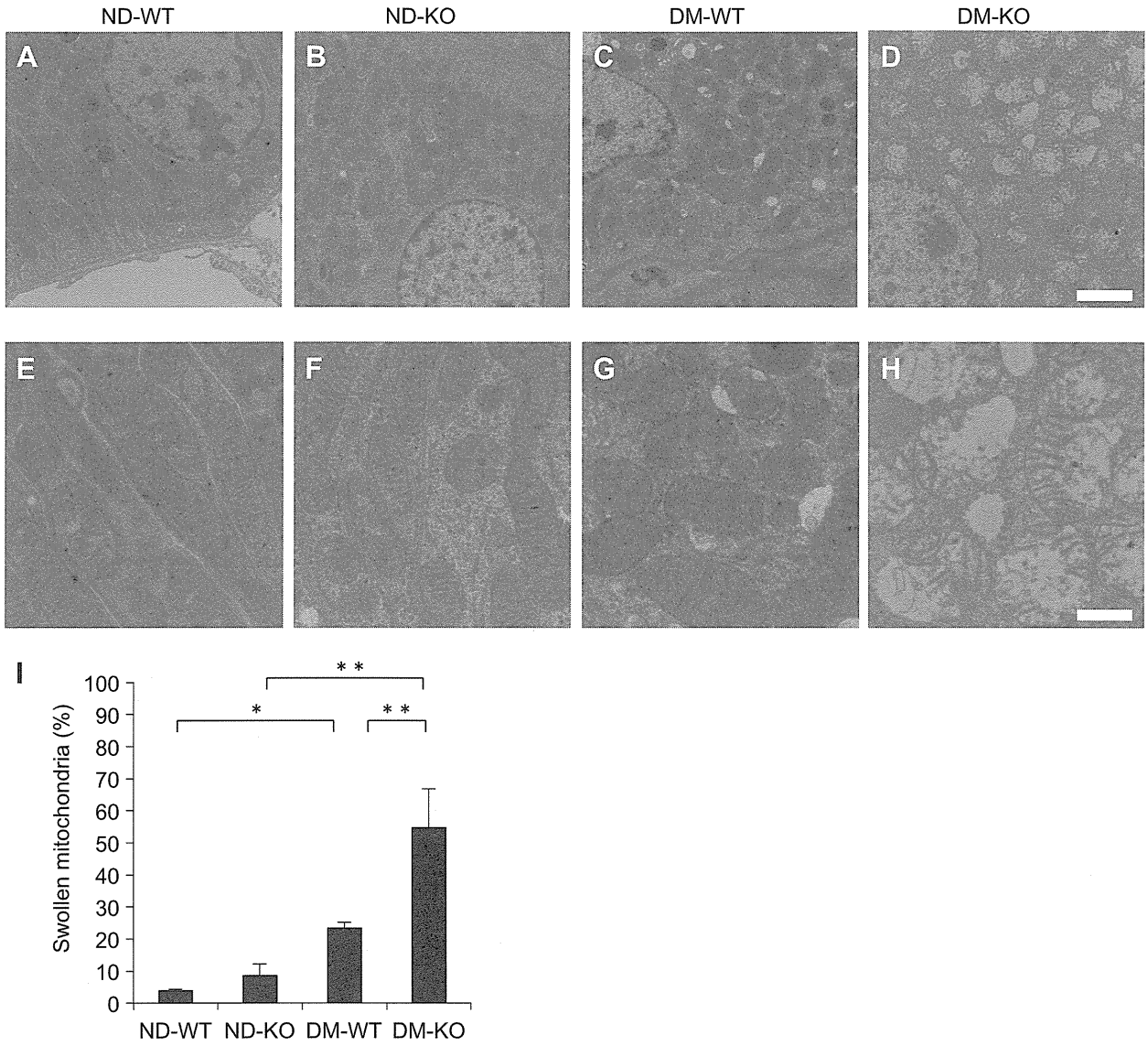


Fig. 6. Representative electron micrographs of proximal tubular epithelial cells in ND-WT (*A* and *E*), ND-KO (*B* and *F*), DM-WT (*E* and *G*), and DM-KO (*D* and *H*) mice. Original magnification: ×1,500 (*top*) and ×4,000 (*bottom*). Swollen mitochondria were increased in proximal tubular epithelial cells of DM-KO mice. Scale bar = 20 μm (*top*) and = 7.5 μm (*bottom*). *I*: quantitative analysis of swollen mitochondria. Swollen mitochondria were evident in diabetic mice and were significantly increased in DM-KO compared with DM-WT. Values are means ± SE. $*P < 0.05$. $**P < 0.01$.

diabetic $MT^{+/+}$ mice. Electron microscopy also showed more severe mitochondrial swelling in proximal tubular epithelial cells in diabetic $MT^{-/-}$ mice compared with diabetic $MT^{+/+}$ mice. It has been reported that mitochondrial morphology is altered in renal diseases including diabetic nephropathy (8, 31, 32). Since MT is a potent antioxidant and adaptive protein that protects cells and tissues from oxidative stress (12), we speculated that MT deficiency is likely to contribute to increased mitochondrial swelling in diabetic $MT^{-/-}$ mice. However, no studies have reported how MT is related to mitochondrial morphology, and thus further studies are needed. We also performed siRNA experiments to explore the effects of MT on the gene expression level of Nox4, as a promoter of ROS generation, and on the mitochondrial ROS in cultured proximal tubular epithelial cells. The fact that Nox4 expression and the intensity of MitoTracker Red CM-H_2XRos were increased by knockdown of MT suggests that MT may prevent Nox4-derived ROS generation by reducing oxidative stress in proximal tubular epithelial cells. Overall, these results indicate that MT deficiency increases diabetes-induced oxidative stress in the interstitium of the kidney.

Inflammation is also associated with the development of diabetic nephropathy (13, 15, 19). The present study revealed that increased expression levels of the macrophage marker CD14, the chemokine MCP-1, and the cytokines TGF-β and OPN noted in diabetic $MT^{+/+}$ mice were further increased in diabetic $MT^{-/-}$ mice. Similarly, macrophage infiltration into the interstitium and interstitial fibrosis were increased in diabetic $MT^{-/-}$ mice compared with diabetic $MT^{+/+}$ mice. However, macrophage infiltration in the glomeruli and mesangial matrix accumulation were similar in both types of mice. Moreover, in vitro studies demonstrated that the expression levels of inflammatory genes including MCP-1, TGF-β, and OPN were increased by knockdown of MT in cultured proxi-

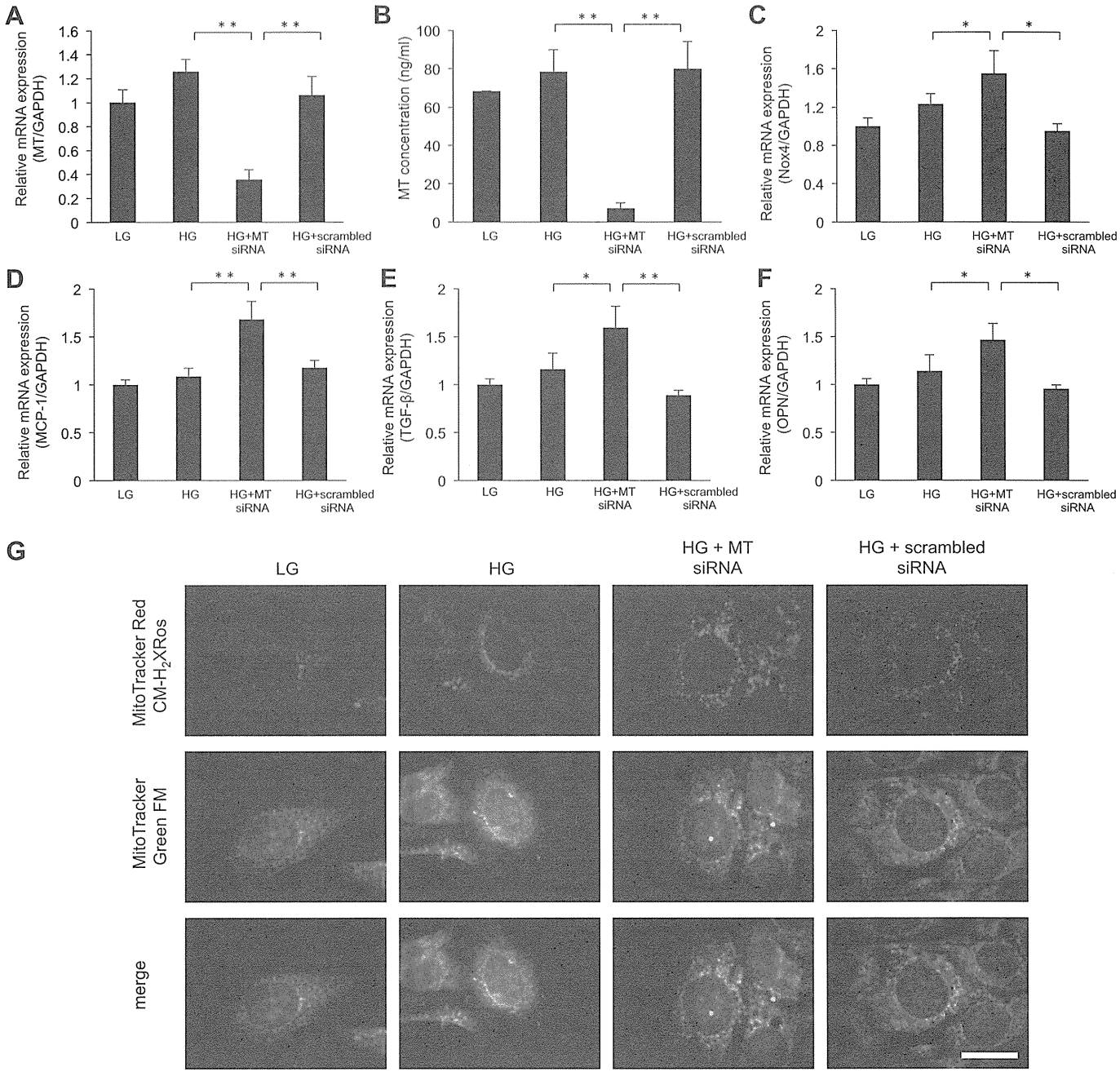


Fig. 7. MT knockdown accelerates high-glucose-induced oxidative stress and inflammation in cultured proximal tubular epithelial cells. *A* and *B*: MT mRNA and protein expression was significantly inhibited in mProx24 cells grown in high-glucose (HG) medium and transfected with MT small interfering (si) RNA compared with cells grown in HG medium and transfected with scrambled siRNA. HG, 25 mM; LG, low-glucose medium (5.5 mM). Values are means ± SE. $^{**}P < 0.01$. *C–F*: quantitative RT-PCR analysis showed that knockdown of MT increased the expression of NADPH oxidase 4 (Nox4) in proximal tubular epithelial cells. MCP-1, TGF-β, and OPN mRNA levels were also increased by MT siRNA, which indicated that knockdown of MT promoted inflammation in proximal tubular epithelial cells. mRNA levels are normalized to GAPDH. HG, 25 mM; LG, 5.5 mM. Values are means ± SE. $^{*}P < 0.05$. $^{**}P < 0.01$. *G*: representative photomicrographs of double immunofluorescent staining using MitoTracker Red CM-H_2XRos and MitoTracker Green FM. The intensity of MitoTracker Red CM-H_2XRos was increased in mProx24 cells transfected with MT siRNA compared with cells transfected with scrambled siRNA. HG, 25 mM; LG, 5.5 mM. Original magnification: ×200.

mal tubular epithelial cells. These results indicate that MT deficiency involves inflammation in the interstitium, but not in the glomeruli, in diabetic nephropathy. The expression of NF-κB, a master regulator of inflammatory genes, is increased in diabetic $MT^{-/-}$ mice compared with diabetic $MT^{+/+}$ mice. This result suggests that MT may suppress high-glucose-induced inflammation by inhibiting NF-κB.

We and others (14, 27) have demonstrated that MT plays a protective role in chronic kidney injury models induced by cadmium or cisplatin in the MT knockout mouse, but no studies have been reported in experimental models of diabetic nephropathy in the MT knockout mouse. Although we have previously shown that MT is upregulated in the kidney of the STZ-induced diabetic rat (21), whether MT prevents diabetes-

induced oxidative stress and inflammation, and thus protects against diabetic nephropathy, remains unclear. Podocyte-specific MT-transgenic mice showed that overexpression of MT in podocytes could ameliorate the primary features of diabetic nephropathy (33), suggesting that protection of the podocytes could inhibit diabetic nephropathy. Induction of renal tubular MT synthesis by zinc supplementation also prevents diabetic nephropathy by acting against oxidative stress (23, 28). Our current and previous study (21) showed that MT is induced mainly in renal tubules, rather than podocytes, in the diabetic kidney. Therefore, MT in renal proximal tubular epithelial cells might thus be a therapeutic target for the treatment of diabetic nephropathy.

In conclusion, we demonstrated that MT deficiency accelerates high-glucose-induced oxidative stress and inflammation in the kidney. The results of this study indicate that MT plays an important role in protecting the kidney from diabetic stress by acting as an antioxidant protein. Our findings suggest that MT might be a novel therapeutic target for the treatment of diabetic nephropathy.

GRANTS

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DISCLOSURES

D. Ogawa and A. Nakatsuka belong to the Department of Diabetic Nephropathy, endowed by Astellas and Boehringer Ingelheim. J. W. is a consultant for Boehringer Ingelheim and receives speaker honoraria from Novartis. H. M. is a consultant for AbbVie and Astellas, receives speaker honoraria from Astellas, MSD, Takeda, and Tanabe Mitsubishi, and receives grant support from Astellas, Daiichi Sankyo, Dainippon Sumitomo, MSD, Novo Nordisk, and Takeda. The authors report no conflicts of interest in this work.

AUTHOR CONTRIBUTIONS

Author contributions: H.T., N.S., M.A., I.M., N.T., T.H., C.S.H., A.N., and H.Y. performed experiments; H.T. and J.E. analyzed data; H.T., D.O., N.S., M.A., I.M., N.T., T.H., C.S.H., A.N., and J.E. interpreted results of experiments; H.T., J.W., and H.Y. prepared figures; H.T. and D.O. drafted manuscript; H.T., D.O., N.S., M.A., I.M., N.T., T.H., C.S.H., A.N., J.E., J.W., H.Y., K.T., and H.M. approved final version of manuscript; D.O. conception and design of research; J.W., H.Y., K.T., and H.M. edited and revised manuscript.

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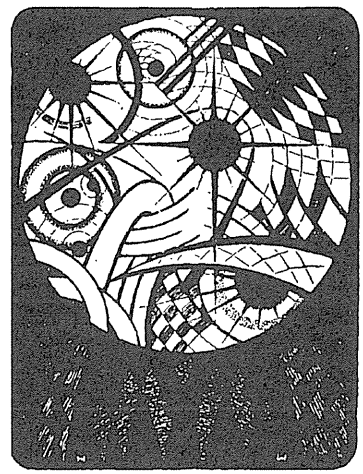

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Predictive Properties of Plasma Amino Acid Profile for Cardiovascular Disease in Patients with Type 2 Diabetes

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Abstract

Prevention of cardiovascular disease (CVD) is an important therapeutic object of diabetes care. This study assessed whether an index based on plasma free amino acid (PFAA) profiles could predict the onset of CVD in diabetic patients. The baseline concentrations of 31 PFAAs were measured with high-performance liquid chromatography-electrospray ionization-mass spectrometry in 385 Japanese patients with type 2 diabetes registered in 2001 for our prospective observational follow-up study. During 10 years of follow-up, 63 patients developed cardiovascular composite endpoints (myocardial infarction, angina pectoris, worsening of heart failure and stroke). Using the PFAA profiles and clinical information, an index (CVD-AI) consisting of six amino acids to predict the onset of any endpoints was retrospectively constructed. CVD-AI levels were significantly higher in patients who did than did not develop CVD. The area under the receiver-operator characteristic curve of CVD-AI (0.72 [95% confidence interval (CI): 0.64–0.79]) showed equal or slightly better discriminatory capacity than urinary albumin excretion rate (0.69 [95% CI: 0.62–0.77]) on predicting endpoints. A multivariate Cox proportional hazards regression analysis showed that the high level of CVD-AI was identified as an independent risk factor for CVD (adjusted hazard ratio: 2.86 [95% CI: 1.57–5.19]). This predictive effect of CVD-AI was observed even in patients with normoalbuminuria, as well as those with albuminuria. In conclusion, these results suggest that CVD-AI based on PFAA profiles is useful for identifying diabetic patients at risk for CVD regardless of the degree of albuminuria, or for improving the discriminative capability by combining it with albuminuria.

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Introduction

Cardiovascular disease (CVD) is a life-threatening complication in patients with diabetes. Since hyperglycemia, hypertension, and dyslipidemia are well recognized as conventional risk factors for CVD, early intervention against them is important to prevent the onset of CVD in this population [1]. Several clinical studies have indicated that the incidence of CVD in patients with type 2 diabetes could be reduced with intensive management for these risk factors [2,3]. The development of biomarkers or an index to identify patients at high risk for CVD is also clinically important as it makes possible the initiation of adequate medication for patients at risk. Excessive urinary albumin excretion, called albuminuria, has been established as a reliable surrogate biomarker for CVD, because an increase or decrease in albuminuria has been reported to directly affect the incidence of CVD [3–5]. Thus, the prevention and reduction of albuminuria by intensive control of the above-mentioned conventional risk factors for CVD is considered an important therapeutic target in the care of patients with diabetes [2,6,7]. Despite these efforts, however, many patients still develop CVD, suggesting that only the evaluation of known risk factors is insufficient to distinguish between patients at high and low risk of CVD. It is therefore important that an additional predictive biomarker or index be found to identify those patients with diabetes who are at risk for CVD.

Recent studies have reported that alteration of plasma metabolomics profiles is significantly associated with certain disease conditions and can predict future development of diseases [8–11]. Among the numerous metabolites, plasma free amino acids (PFAAs) may be potent metabolites that have potential as excellent disease biomarkers because circulating free amino acids are involved in protein synthesis, organ networks, and as metabolic regulators of physiological states [12]. Recent technological advances have made possible the highly accurate analysis of PFAA levels using high-performance liquid chromatography-electrospray ionization-mass spectrometry (HPLC-ESI-MS) [13].