A

	Control	Diabetes
n	6	8
Body weight (g)	31.2 ± 7.2	25.8 ± 6.2
Blood glucose (mg/dl)	168 ± 32	558 ± 76*

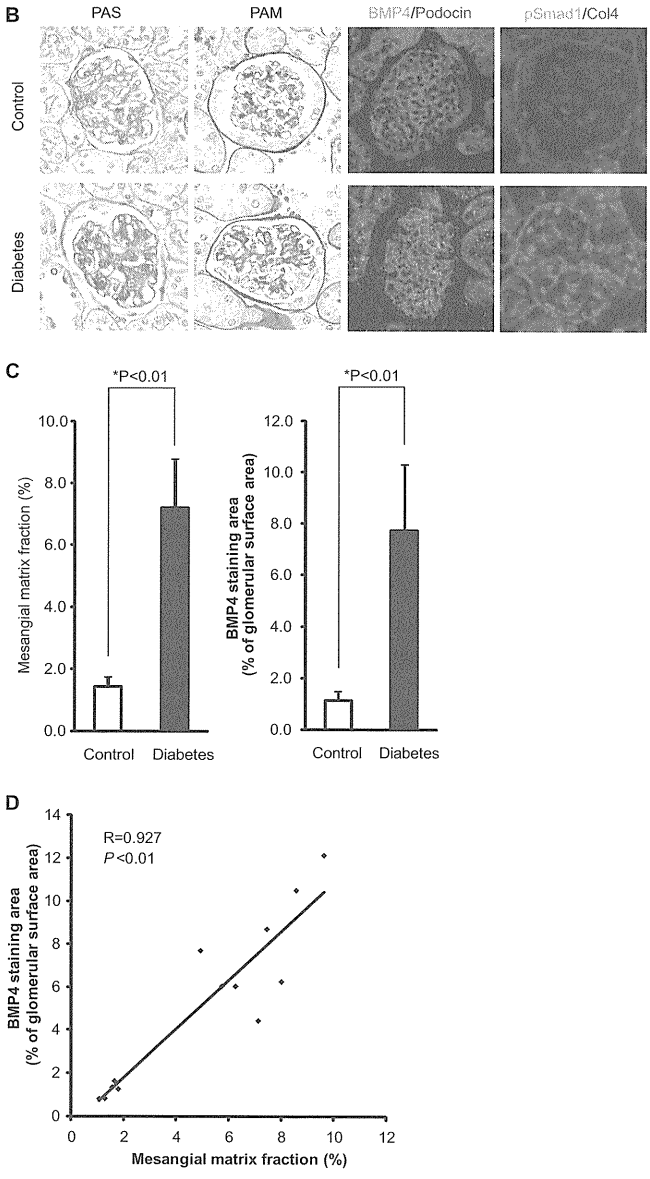


FIGURE 3. **Morphological changes in experimental models of diabetic nephropathy.** *A,* biochemical data in *Control* (nondiabetic mouse) and *Diabetes* (STZ injection mouse) ($n = 6$ for Control; $n = 8$ for Diabetes; results expressed as mean ± S.D. *, $p < 0.01$, *t* test). *B,* representative light microscopy and immunohistochemistry for Control (*upper panels*) and Diabetes (*lower panels*) after 20 weeks of STZ or citrate buffer injection. *PAM*, periodic acid methenamine. *C,* mesangial sclerotic fraction was determined as percentage of mesangial matrix area per total glomerular surface area. The expression of Bmp4 was determined as percentage of Bmp4-positive staining area per total glomerular surface area ($n = 6$ for control; $n = 8$ for Diabetes; $p < 0.01$, *t* test). *D,* correlation between glomerular Bmp4-positive area and mesangial matrix fraction ($R = 0.927$; *, $p \leq 0.01$, *t* test).

also suppressed by *Alk3* silencing in mouse MCs (Fig. 2*C*). These findings implicate that Bmp4 and its receptor Alk3 affect the activation of Smad1 and Col4 expression in mouse MCs.

Bmp4 Is Induced in the Glomeruli of Diabetic Mice—We next investigated whether the Bmp4-Smad1 signaling pathway acts *in vivo* by using experimental models of DN. STZ mice exhibited glomerulosclerosis (Fig. 3*B*). A positive staining of Bmp4 was observed in expanded mesangial areas, which was merged with Col4 but not Podocin (Fig. 3*B*). The pSmad1 expression was also positive with a nucleic pattern in the glomeruli, indicating activation of the Smad1 signaling pathway in diabetic nephropathy mice (Fig. 3*B*), although neither were detected in control mice. The increase in glomerulosclerotic areas detected by silver staining (periodic acid methenamine) was remarkable in STZ mice (7.2 ± 1.5%) compared with wild type mice (1.4 ± 0.3%) (Fig. 3*C*). Immunohistochemical positivity of Bmp4 levels was observed in accordance with the levels of glomerulosclerosis (correlation factor $r = 0.927$, $p < 0.01$) (Fig. 3*D*). Taken together, Bmp4 may contribute to Smad1 activation and subsequent overproduction of ECM proteins in these experimental models of DN. Hence, we propose the hypothesis that Bmp4 plays a critical role in the development of renal changes that are characteristic of DN.

Generation of Inducible Bmp4 Transgenic Mice by the Tamoxifen-regulated Cre-loxP System—To examine whether Bmp4 is responsible for the formation of characteristic renal changes and albuminuria in the development of DN, we generated transgenic mice with inducible expression of *Bmp4* using the tamoxifen-regulated Cre/LoxP system in a nondiabetic condition (Fig. 4*A*). It is well known that Bmp4 is a multifunctional signaling molecule required for normal embryonic development. Because no specific promoter-directed expression in MCs has been developed, we decided to use the CMV enhancer and β-actin promoter, which has been reported to be activated only in glomeruli but not in tubules, arteries, and interstitium (27). We confirmed that the CMV/actin promoter drives the transgene expression predominantly in the whole glomeruli by monitoring GFP expression (Fig. 4*C*). To discuss the direct interaction of Bmp4 induction and pathophysiological changes in this inducible transgenic mouse system, we set several negative controls to exclude the possible nonspecific factors as follows: environmental and feeding conditions, insertion of the transgenes in the genome, and pharmacological actions of tamoxifen and nuclear translocation of MCM. The *Bmp4* transgenic mice did not have any abnormalities before induction with tamoxifen as we show in the *upper row* of Fig. 5*A*, which indicates that neither environmental and feeding conditions nor insertion of the transgenes in the genome has any significant effect on their health. Additionally, the *MCM* transgenic mice did not have any obvious symptoms after they were fed with tamoxifen-containing diet (data not shown), even though translocation of activated MCM into nuclei takes place. These results indicate that both pharmacological actions of tamoxifen and nuclear translocation of the MCM did not have significant effects. Therefore, we concluded that pathophysiological changes in the *Bmp4* transgenic mice described below are a direct consequence of induced Bmp4 overexpression.

Induced Overexpression of Bmp4 in the Glomeruli of Adult Mice Leads to Glomerulosclerosis and Advanced Albuminuria—Tamoxifen-inducible *Bmp4* transgenic mice revealed extensive expansion of the mesangial matrix, compared with noninducible glomeruli. To quantify the extent of mesangial expansion, we used an image processor for analytical pathology system,

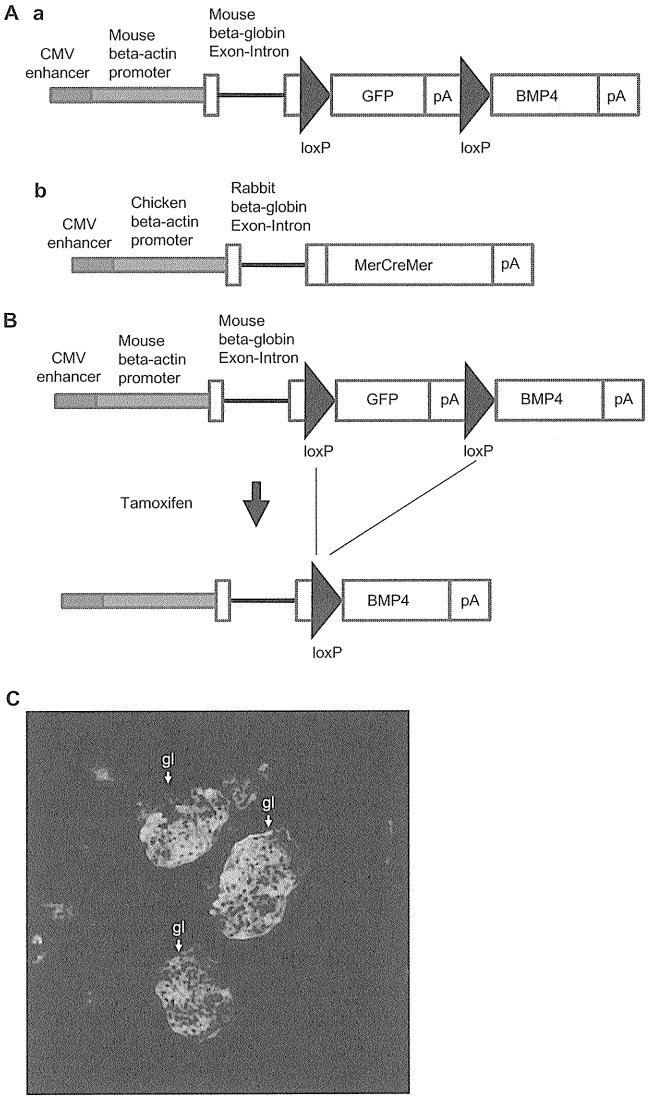


FIGURE 4. **Experimental overview of the generation of tamoxifen-inducible *Bmp4* transgenic mice.** *A, panel a,* transgene for inducible murine *Bmp4* (*pMac II*-floxed *GFP-Bmp4*) was constructed using an expression vector for mammalian cells, pMac II. pMacII is composed of a CMV enhancer and mouse β-actin promoter as transcription regulatory elements, followed by the genomic DNA of mouse β-globin for appropriate splicing of the transgene and addition of the poly(A) signal. *Renilla reniformis* GFP cDNA with mouse β-globin poly(A) signal flanked by directly repeated *loxP* sequences (*loxP-GFP-pA-loxP*) was inserted into the cloning site of pMac II. Finally, a full length of murine *Bmp4* cDNA with Kozak sequence (GCCACC) was inserted downstream from the *loxP-GFP-pA-loxP*. *Panel b,* tamoxifen-inducible cre recombinase, MCM, is a site-specific recombinase cre fused with two ligand-binding domains of a mutated murine estrogen receptor. The *MCM* expression vector was constructed by inserting the *MCM* cDNA into the pCAGGS. *B,* for induction of *Bmp4* gene expression, 8-week old double transgenic mice were fed a diet (CE-2, CLEA Japan) containing 0.02% tamoxifen citrate (Sigma). *C,* GFP expression area displayed predominance at the glomeruli (*gl*).

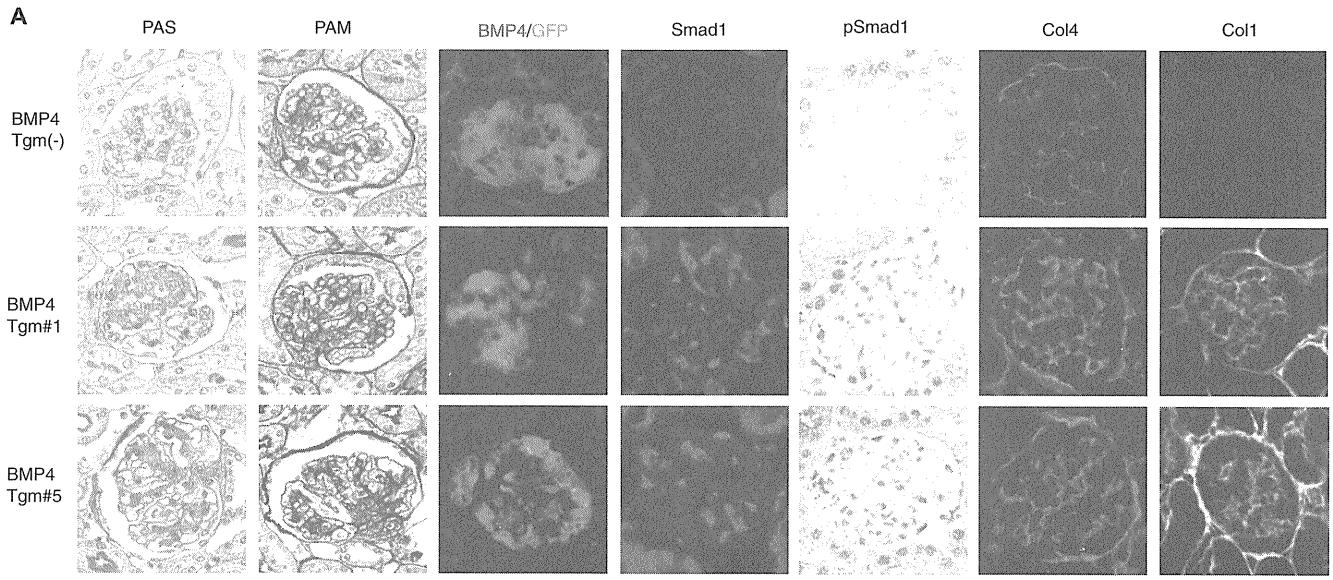
which showed an increase in the mesangial matrix fraction compared with noninducible mice (Fig. 5, *A* and *B*). These transgenic mice also showed significant induction of glomerular expressions of Smad1, pSmad1, Col4, and Col1 compared with noninducible mice (Fig. 5*A*). Furthermore, the *Bmp4* tgm exhibited marked thickening of the GBM as well as mesangial expansion in electron microscopic analyses (Fig. 5, *C* and *D*), both of which are characteristic of human DN. Albuminuria was dramatically increased in inducible *Bmp4* tgm compared with noninducible mice. Moreover, the degree of expansion of mesangial areas in inducible *Bmp4* tgm was significantly correlated with the degree of albuminuria (Fig. 5, *E* and *F*). These changes were similarly observed in another line of *Bmp4* tgm. Collectively, inducible *Bmp4* tgm were able to mimic diabetic changes in glomeruli by exhibiting pathological features remarkably resembling human DN in a nondiabetic condition.

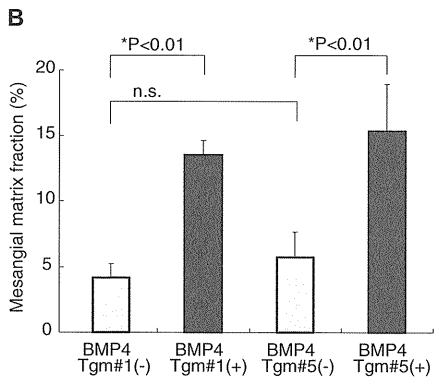
Heterozygous Bmp4 Knock-out Mice Exhibited Attenuation of Glomerulosclerosis in Diabetes—Although forced expression of Bmp4 in glomeruli caused renal changes similar to DN, there still remains the possibility that *Bmp4* tgm showed renal changes of different renal diseases. Therefore, we investigated whether the reduction of Bmp4 expression improved the diabetic glomerular changes by using heterozygous *Bmp4* knock-out mice. Blood glucose was increased, and body weight was decreased in both diabetic mouse groups compared with the control mouse group (Fig. 6*A*). We observed the attenuation of mesangial matrix expansion in diabetic $Bmp4^{+/-}$ mice compared with diabetic wild type mice (Fig. 6*B*). Furthermore, the pSmad1 expression was decreased in $Bmp4^{+/-}$ STZ mice compared with C57BL/6 STZ mice (Fig. 6*B*). We also found that glomerular expression of Col4 was reduced in diabetic $Bmp4^{+/-}$ mice, compared with diabetic wild type mice (6.3 ± 2.4% in $Bmp4^{+/-}$ STZ mice *versus* 9.7 ± 0.7% in C57BL/6 STZ mice, $p < 0.05$) (Fig. 6*C*). These data suggest that BMP4 is a critical determinant for the development of DN.

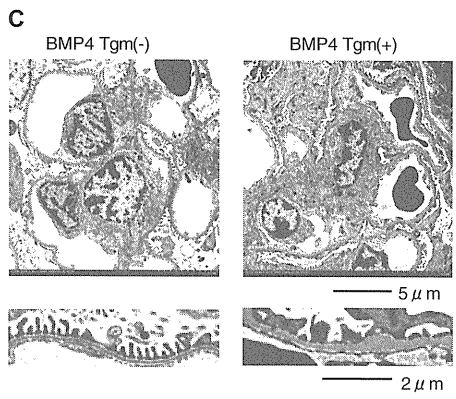
DISCUSSION

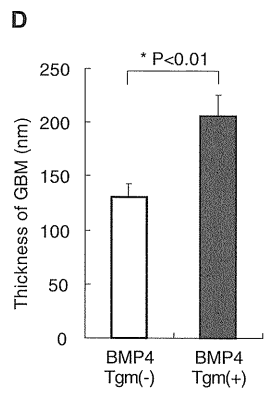
DN is the leading cause of end-stage renal disease and a major contributing cause of morbidity and mortality in patients with diabetes throughout the world. Typical features include thickening of the GBM and mesangial matrix hyperplasia (so-called glomerulosclerosis) (28). Only the latter pathological finding is related to the decrease in the glomerular filtration rate (29), which is the general clinical event for renal damage in type 1 and 2 diabetes mellitus. There are several factors associated with the development of DN, including AGE, protein kinase C, oxidative stress, and an increase in glomerular hemodynamics. Among them, we have recently reported that Smad1 is induced and phosphorylated in diabetic kidneys, but the factors driving the induction as well as phosphorylation of Smad1 in diabetes mellitus have remained elusive. Here, we provide the first direct evidence that the BMP4 signaling pathway may play a crucial role in the development of diabetic glomerulosclerosis.

Prolonged exposure to hyperglycemia is now recognized as the principal causal factor of diabetic complications (30, 31). Its deleterious effects are attributable to the formation of sugar-derived protein adducts and cross-links known as AGE. Exposure of cultured mesangial cells to AGE results in a receptor-mediated up-regulation of mRNA and protein secretion of Col4 (4, 32). We have previously shown that AGE/RAGE system is critical for progression of glomerulosclerosis, as shown in the study AGE breaker and *RAGE* knock-out mice (25, 33–35). However, there is little information regarding the downstream









E

	BMP4 Tgm(-)	BMP4 Tgm(+)
n	6	7
Albuminuria (mg/g·CRE)	12.9 ± 3.5	242.1 ± 295.0

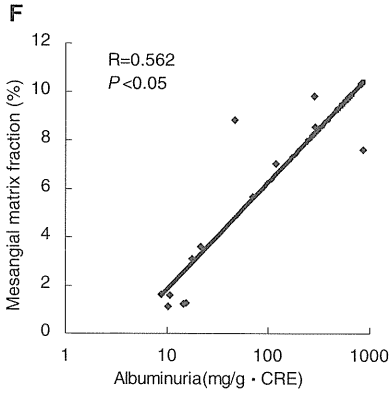
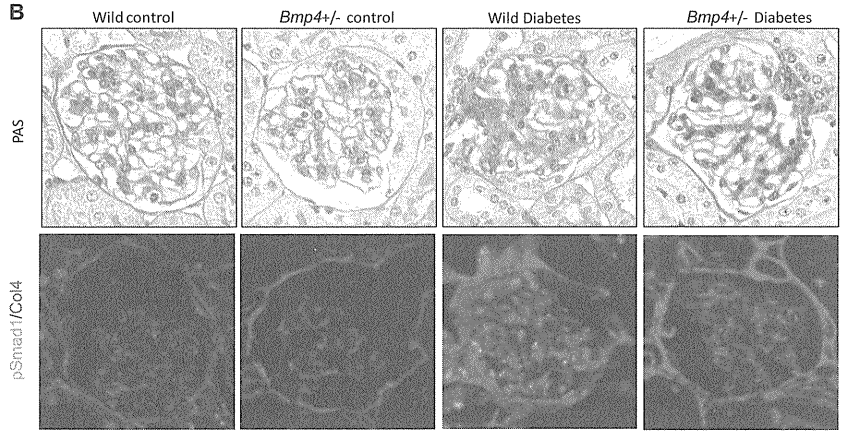


FIGURE 5. **Morphological changes in *Bmp4* tgm glomeruli.** *A,* representative light microscopy and immunohistochemistry pictures of glomeruli. The *upper row* is an uninduced mouse, and the *middle* and *lower rows* are two representative *Bmp4* tgm at 5 days after the induction. *B,* mesangial sclerotic area ($n = 6$ for control; $n = 7$ for *Bmp4* tgm; $p < 0.01$, *t* test, *n.s.*, not significant). *C,* electron micrographs of glomeruli in uninduced (*left panels*) and induced (*right panels*) *Bmp4* tgm. *Upper panels* show low magnification (*bar,* 5 μm), although lower panels show high magnification (*bar* = 2 μm). *D,* thickness of the GBM measuring 50 sections at three different sites. Areas of wrinkled GBM were excluded. The mean thickness of the GBM was 130 ± 12 nm in noninduced mice and 206 ± 20 nm in induced mice ($p < 0.01$, *t* test). *E,* albuminuria in each group ($n = 6$ for control; $n = 7$ for *Bmp4* tgm; results expressed as means ± S.D.). *F,* correlation between albuminuria and mesangial matrix fraction ($R = 0.562$, $p \leq 0.05$, *t* test).

A

	wt control	*Bmp4+/- control*	wt Diabetes	*Bmp4+/-* Diabetes
n	6	6	8	7
Body weight (g)	31.2 ± 7.2	29.7 ± 1.8	25.8 ± 6.2	17.6 ± 3.9
Blood glucose (mg/dl)	168 ± 32	168 ± 18	558 ± 76	530 ± 70



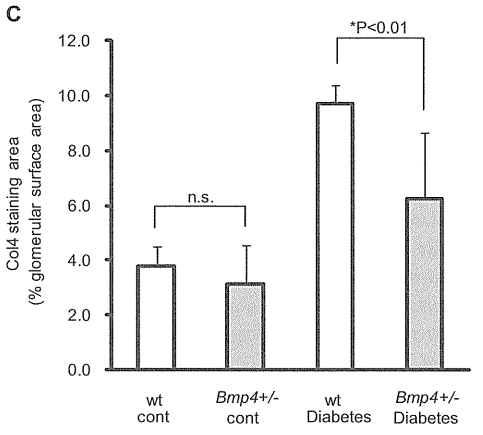


FIGURE 6. **Morphological changes in diabetic *Bmp4*$^{+/-}$ mice.** *A*, biochemical data in each group. *Bmp4*$^{+/+}$ control = C57BL/6 nondiabetic mouse; *Bmp4*$^{+/-}$ control = *Bmp4*$^{+/-}$ nondiabetic mouse; *Bmp4*$^{+/+}$ *Diabetes* = C57BL/6 diabetic mouse; *Bmp4*$^{+/-}$ *Diabetes* = Bmp4$^{+/-}$ diabetic mouse; results expressed as mean ± S.D. *B*, representative light microscopy (*upper panels*) and immunohistochemistry of pSmad1 and Col4 (*lower panels*) for *Bmp4*$^{+/+}$ control, *Bmp4*$^{+/-}$ control, *Bmp4*$^{+/+}$ *Diabetes*, and *Bmp4*$^{+/-}$ *Diabetes* at after 20 weeks of STZ or citrate buffer injection. *C*, expansion of mesangial fraction was determined as percentage of Col4-positive area per total glomerular surface area ($p < 0.01$, *t* test).

signal transduction of the AGE-RAGE axis in DN. Previous studies have shown that TGF-β increases the levels of ECM protein in DN (36–38). It is generally known that Smad3 functions as a key intracellular signal transducer for profibrotic TGF-β responses in various cells. Although the role of the Smad3 pathway in the pathogenesis of DN has only been demonstrated for interstitial fibrosis in a model of obstructive nephropathy (39), the interruption of Smad3 signaling did not improve albuminuria in STZ-diabetic *Smad3* knock-out mice (40). Similarly, albuminuria failed to improve in diabetic db/db mice treated with an anti-TGF-β antibody (41). These results suggest the existence of another signaling pathway involved in the development of DN. Here, we first confirmed that *RAGE* knockdown leads to the decreased expression of Bmp4 and the subsequent decrease of ECM production. In addition, we demonstrated that Bmp4 not only phosphorylated but also directly induced Smad1 in mesangial cells. Because it has been shown that Smad1 is absent in the normal glomeruli of adult mice and humans (6, 42), Bmp4 may be involved in the initiation of glomerulosclerosis. Expression of Bmp4 levels were also correlated with the severity of glomerulosclerosis in STZ mice. Therefore, there is a possibility that the extent of induction of BMP4 via AGE-RAGE signal correlates with pathological changes in glomeruli in DN.

The BMP family of proteins belongs to the TGF-β superfamily; these proteins exert their effects through association with two different types of serine/threonine kinase receptors, type I and type II receptors (43–45). The type IA receptor (BMPR-IA/ALK3) and BMPR-IB/ALK6 share a high similarity and specifically bind several BMP family members. First, we confirmed that Smad1 was phosphorylated through Alk3, but not Alk6, and induced the expression of Col4. We then investigated the possibility that Bmp2 and Bmp4, which originate from a single ancestral gene highly similar to the fly *dpp* gene (46, 47) and share greater than 90% amino acid homology in the ligand domain, may influence the Smad1-induced Col4 expression. Notably, the inducible *Bmp4* transgenic mice exhibited remarkable glomerulosclerosis and albuminuria, and Smad1 activation and elevated expression of Col1 and Col4 were observed in the glomeruli. These findings in the kidney are all consistent with the features of diabetic glomerulosclerosis. Therefore, forced expression of Bmp4 in glomeruli was able to mimic DN in a nondiabetic condition, strongly suggesting that Bmp4 plays a central role for the development of DN. Moreover, from the point of view of a therapeutic approach to DN, we induced diabetes on heterozygous *Bmp4* knock-out mice and analyzed them. Diabetic heterozygous *Bmp4* knock-out mice exhibited the reduction of glomerular changes compared with diabetic wild type mice, suggesting that the expression levels of Bmp4 correlates with the degree of expansion of mesangial areas in diabetes. In addition, a recent report demonstrates that Col4 itself contributes to acceleration of ECM expansion through the direct regulatory effect of Col4 on Bmp4 signaling (48). Accordingly, the activation of the BMP4 signaling pathway and overproduction of COL4 may cooperatively contribute to the progression of diabetic glomerulosclerosis. Taken together, these results provide direct and specific evidence for the causal role of BMP4 in the pathogenesis of DN *in vivo*. Because the BMPs are a large family of proteins that share common structural features, highly specific inhibitors or neutralizing antibodies for BMP4 would offer us novel therapeutic tools to suppress the progression of diabetic nephropathy.

In summary, we have clearly shown that the activation of BMP4 signal transduction through Smad1 may play an essential role for the initiation and progression of diabetic changes in the kidney. Preferential inhibition of the BMP4 signaling pathway

may provide a novel therapeutic approach to DN without undesirable adverse effects, because, for instance, BMP2 might have a protective effect against renal damage (49).

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Concentration-dependent Dual Effects of Hydrogen Peroxide on Insulin Signal Transduction in H4IIEC Hepatocytes

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Abstract

Background: Oxidative stress induced by the accumulation of reactive oxygen species (ROS) has a causal role in the development of insulin resistance, whereas ROS themselves function as intracellular second messengers that promote insulin signal transduction. ROS can act both positively and negatively on insulin signaling, but the molecular mechanisms controlling these dual actions of ROS are not fully understood.

Methodology/Principal Findings: Here, we directly treated H4IIEC hepatocytes with hydrogen peroxide (H2O2), a representative membrane-permeable oxidant and the most abundant ROS in cells, to identify the key factors determining whether ROS impair or enhance intracellular insulin signaling. Treatment with high concentrations of H2O2 (25–50 μM) for 3 h reduced insulin-stimulated Akt phosphorylation, and increased the phosphorylation of both JNK and its substrate c-Jun. In contrast, lower concentrations of H2O2 (5–10 μM) enhanced insulin-stimulated phosphorylation of Akt. Moreover, lower concentrations suppressed PTP1B activity, suggesting that JNK and phosphatases such as PTP1B may play roles in determining the thresholds for the diametrical effects of H2O2 on cellular insulin signaling. Pretreatment with antioxidant N-acetyl-L-cysteine (10 mM) canceled the signal-promoting action of low H2O2 (5 μM), and it canceled out further impairment of insulin of insulin signaling induced by high H_2O_2 (25 μM).

Conclusions/Significance: Our results demonstrate that depending on its concentration, H2O2 can have the positive or negative effect on insulin signal transduction in H4IIEC hepatocytes, suggesting that the concentration of intracellular ROS may be a major factor in determining whether ROS impair or enhance insulin signaling.

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Introduction

Insulin resistance is an underlying problem in people with type 2 diabetes and metabolic syndrome [1]. In an insulin-resistant state, impaired insulin action promotes hepatic glucose production and reduces the uptake of glucose by peripheral tissues, resulting in systemic hyperglycemia. In addition to type 2 diabetes and metabolic syndrome, the development of various other diseases such as non-alcoholic steatohepatitis [2] and atherosclerosis [3] involves insulin resistance. It is commonly assumed that combating insulin resistance is a viable therapeutic strategy in several kinds of diseases, although the molecular mechanisms underlying insulin resistance are not fully understood.

Oxidative stress induced by the accumulation of reactive oxygen species (ROS) has a causal role in the development of insulin resistance. Using models in which cells were treated with tumor-necrosis factor α and glucocorticoids, Houstis et al. showed that increased ROS levels are an important trigger for insulin resistance in numerous contexts [4]. Activation of stress kinases such as C-Jun N-terminal kinase (JNK) and IκB kinase contributes to insulin resistance associated with oxidative stress [5]. In a previous report, we demonstrated that treatment with palmitate, a C16:0 saturated fatty acid, induces insulin resistance in H4IIEC hepatocytes by stimulating the generation of ROS in the mitochondria and thereby, the activation of JNK [6]. The administration of antioxidants such as N-acetylcysteine and α-tocopherol partially rescued cells from palmitate-induced insulin resistance, suggesting that antioxidative therapy may be useful in attenuating insulin resistance in patients with type 2 diabetes or metabolic syndrome.

A growing body of evidence suggests that ROS function as intracellular second messengers to promote signaling by hormones, including insulin. Goldstein et al. have shown that insulin-induced endogenous hydrogen peroxide enhances proximal and distal insulin signaling, at least partly through the oxidative inhibition of protein tyrosine phosphatase 1B (PTP1B), which negatively regulates insulin

action [7]. More recently, Loh et al. reported that mice lacking glutathione peroxidase 1 (Gpx1), a key enzyme involved in the removal of ROS, are protected from high-fat diet-induced insulin resistance, providing causal evidence for the enhancement of insulin signaling by ROS *in vivo* [8]. These early reports indicate that ROS can act both positively and negatively on insulin signaling. However, the molecular mechanisms regulating the dual actions of ROS on insulin signaling are not fully understood.

In the current study, to identify the key factors determining whether ROS impair or enhance intracellular insulin signaling, we directly treated H4IIEC hepatocytes with hydrogen peroxide (H_2O_2), a representative membrane-permeable oxidant and the most abundant ROS in the cell. Our results demonstrate that H_2O_2 has dual effects on insulin signal transduction in H4IIEC hepatocytes and that these roles depend on the H_2O_2 concentration used, suggesting that the intracellular concentration of ROS themselves may be a major factor in determining whether ROS impair or enhance insulin signaling.

Results

Time course of extracellular H_2O_2 concentration following its administration to H4IIEC hepatocytes

We treated H4IIEC hepatocytes with H_2O_2, a representative membrane-permeable ROS. Exogenous H_2O_2 is time-dependently reduced and neutralized by intracellular antioxidant enzymes. To assess how long the H_2O_2 remained effective, we measured H_2O_2 concentrations in the culture medium over a specific time course after its administration (Fig. 1). The H_2O_2 concentration in the culture medium returned to basal level within 30 min of its administration to H4IIEC hepatocytes. Therefore, when cells were treated with H_2O_2 for a total of 3 h in subsequent experiments, the H_2O_2-containing medium was replaced every 30 min.

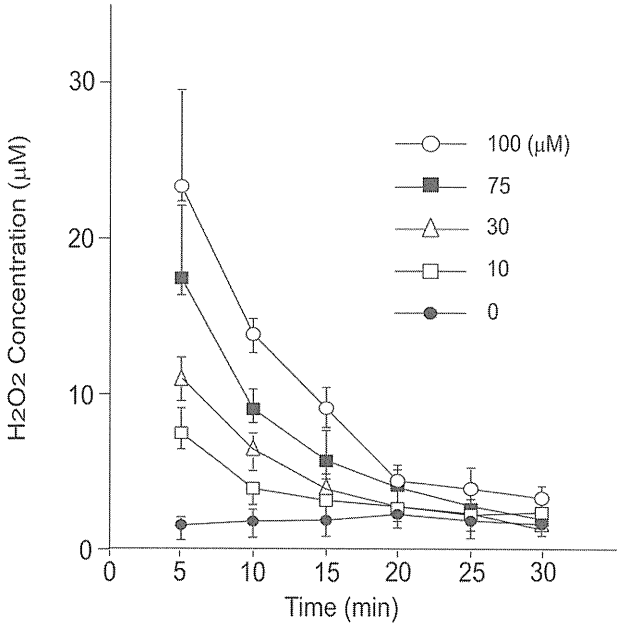

Figure 1. Time course of extracellular H2O2 concentration following its administration to H4IIEC hepatocytes. H4IIEC cells were incubated with the indicated concentrations of H2O2 for the indicated periods of time, and then the concentration of H2O2 in the culture medium was measured by ferrous oxidation of xylenol orange (FOX) assay.
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Dual effects of H_2O_2 on insulin-stimulated phosphorylation of Akt and GSK-3

We examined the effects of H_2O_2 on insulin signaling in H4IIEC hepatocytes (Fig. 2). H4IIEC3 cells were treated with a range of H_2O_2 concentrations for 3 h and then stimulated with insulin for 15 min. As expected, insulin-stimulated serine phosphorylation of Akt and GSK-3α was inhibited at high concentrations of H_2O_2. Insulin-stimulated tyrosine phosphorylation of the insulin receptor (IR) was unaffected by high H_2O_2 concentrations. In contrast, lower concentrations of H_2O_2 enhanced insulin-stimulated phosphorylation of Akt and GSK-3α, without affecting the overall levels of the proteins. We found that 5–10 μM were the concentrations of which H_2O_2 certainly promotes insulin-induced Akt phosphorylation in our cell lines. Thus, we used 5–10 μM of H_2O_2 to certainly increase insulin signaling in the following experiments.

Effects of H_2O_2 treatment on the expression of genes encoding antioxidant enzymes

We hypothesized that weak oxidative stress caused by low concentrations of H_2O_2 may trigger a preconditioning response by inducing antioxidant enzymes such as catalase and Gpx. We assayed the expression of the genes encoding catalase, Gpx1, and Gpx3 in H4IIEC hepatocytes 3 h after treatment with various concentrations of H_2O_2 (Fig. 3). Treatment with H_2O_2 at concentrations in the range 5–50 μM did not alter the expression of any of these three genes.

Attenuation of PTP1B activity contributes to H_2O_2-induced enhancement of insulin signaling

To clarify the mechanism by which low concentrations of H_2O_2 enhance insulin signaling, we measured the activity of PTP1B, a negative regulator of insulin signaling, in H_2O_2-treated cells. PTP1B activity was dose-dependently suppressed by H_2O_2 (Fig. 4A), even at H_2O_2 concentrations as low as 5 μM. Overall PTP1B protein levels were unaffected by H_2O_2 treatment (Fig 4B). To further determine whether phosphatases mediate the insulin-promotive effect induced by low concentrations of H_2O_2, we treated the cells with vanadate, a non-specific inhibitor for phosphatases such as PTP1B (Fig. 5). Coadministration with vanadate increased insulin-stimulated Akt phosphorylation in the cells treated with 5 μM of H_2O_2. However, it did not further increase insulin-stimulated Akt phosphorylation in the presence of 10 μM of H_2O_2 (Fig. 5).

Activation of JNK contributes to H_2O_2-induced insulin resistance

To examine the mechanism by which high concentrations of H_2O_2 impair insulin signaling, we measured the levels of phospho-JNK in H_2O_2-treated cells. JNK is a stress-activated protein kinase that phosphorylates IRS-1 and -2 at serine residues in response to increases in cellular ROS levels. JNK-induced serine phosphorylation of IRS impairs IRS tyrosine phosphorylation, resulting in the inhibition of insulin receptor-mediated signaling. Treatment with H_2O_2 increased the phosphorylation of both JNK and its direct target, c-Jun dose-dependently (Fig. 6). To further determine whether JNK mediates insulin resistance induced by high concentrations of H_2O_2, we transfected H4IIEC hepatocytes with siRNA specific for JNK1 (Fig. 7). Knockdown of JNK partly suppressed serine phosphorylation of IRS-1 induced by high concentrations of H_2O_2 (Figure 7A). As a result, insulin resistance induced by high concentrations of H_2O_2 was partly rescued by silencing of JNK (Fogiure 7B). These results suggest that high

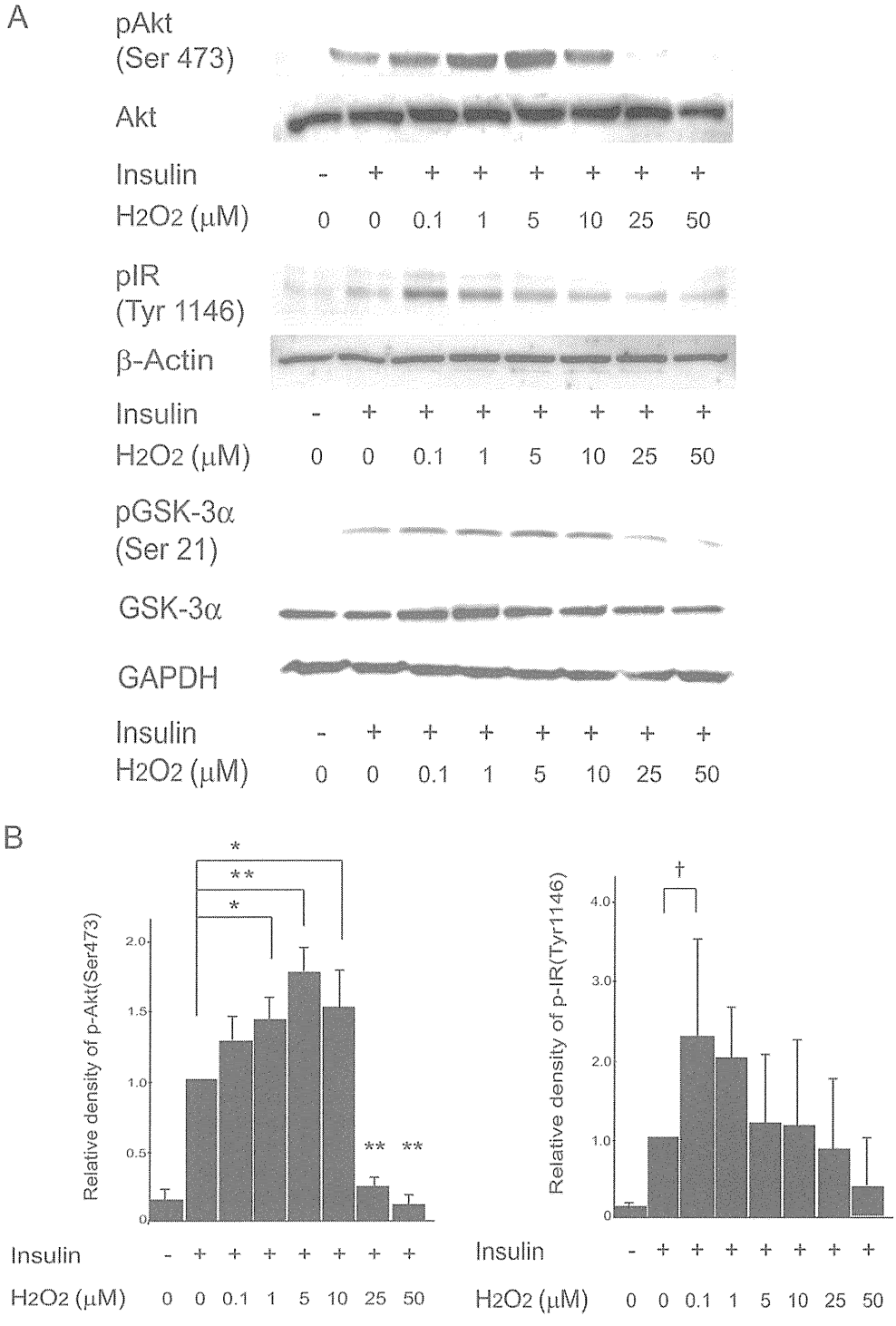

Figure 2. Effects of H2O2 on insulin-stimulated tyrosine phosphorylation of the IR and serine phosphorylation of Akt and GSK-3 in H4IIEC hepatocytes. (A) H4IIEC cells were serum-starved overnight and then treated with the indicated concentrations of H2O2 for total 3 h. Then, the cells were stimulated with insulin (1 ng/mL) for 15 min. Total cell lysates were resolved by SDS-PAGE, transferred to a PVDF membrane, and probed using the indicated antibodies. Signals were detected by chemiluminescence. Representative blots are shown. (B) Quantitative data from densitometric analysis of blots from three (p-IR) and four (p-Akt) independent experiments, respectively. Relative density is mean fold increase over control ± S.E.M. * $p<0.05$, versus treatment with insulin alone; ** $p<0.01$, versus treatment with insulin alone.
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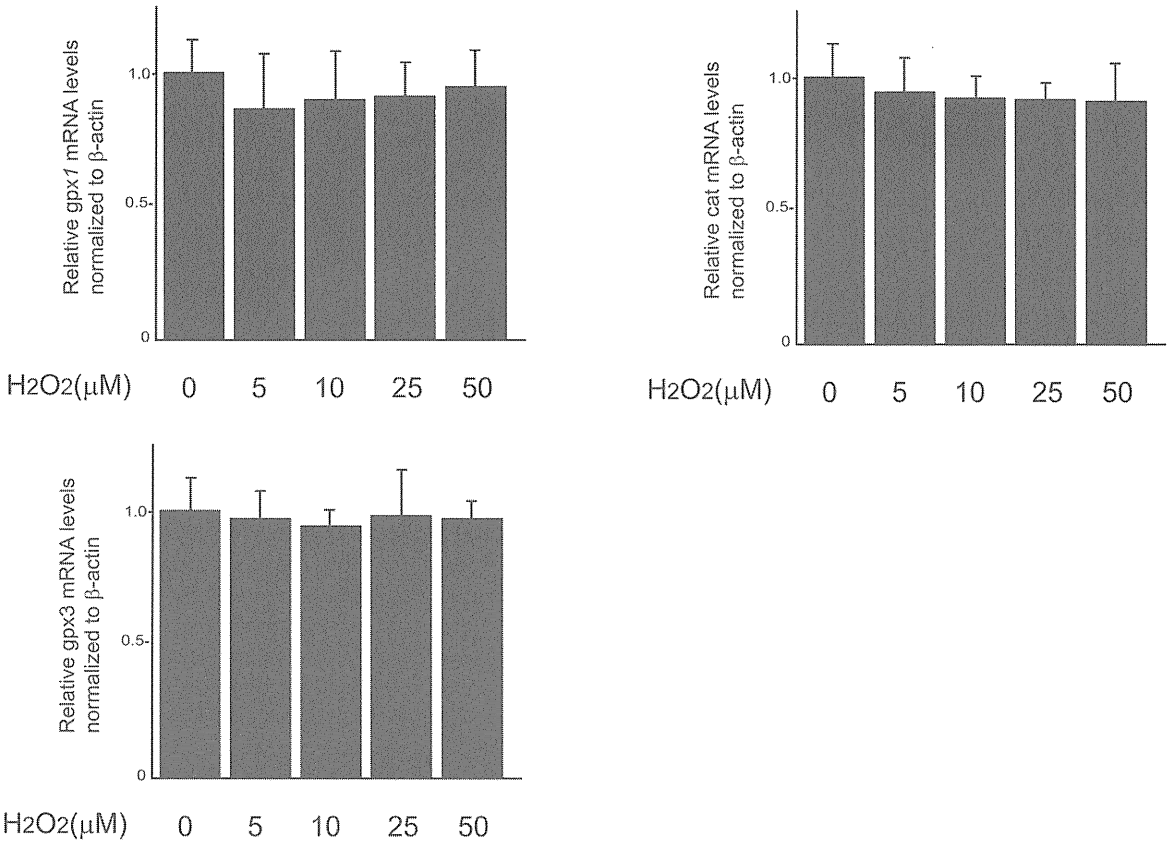

Figure 3. Effects of H_2O_2 on the expression of mRNAs encoding antioxidative enzymes in H4IIEC hepatocytes. H4IIEC cells were serum-starved overnight and treated with the indicated concentrations of H_2O_2 for 3 h. Levels of the mRNAs encoding GPX1, GPX3, and catalase were measured by real-time-PCR. Values represent the mean ± S.D ($n=6$).
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concentrations of H_2O_2 reduce insulin-stimulated Akt phosphorylation, at least partly, by increasing the phosphorylation of JNK and serine residue of IRS-1.

Effects of antioxidant N-acetyl-L-cysteine on H_2O_2-induced alterations of insulin signal transduction in H4IIEC hepatocytes

Next, we determined whether antioxidant N-acetyl-L-cysteine (NAC) attenuates the dual actions of H_2O_2 on insulin signal transduction. We pretreated H4IIEC hepatocytes with 10 mM of NAC, which corresponds to the maximum concentration of NAC commonly used in *in vitro* experiments [6]. Pretreatment with NAC decreased H_2O_2 concentrations in the culture medium of the cells treated with H_2O_2 (Fig. 8). NAC at the concentration of 10 mM was enough to quench H_2O_2 at up to 50 μM. NAC canceled the signal-promoting action of low concentrations of H_2O_2 (Fig. 9A). In addition, although NAC impaired insulin-stimulated phosphorylation of Akt in the absence of H_2O_2, it canceled out further impairment of insulin signaling induced by 25 μM of H_2O_2 (Fig. 9B).

Discussion

In the current study, we used the membrane-permeable oxidant H_2O_2 to demonstrate that ROS can exert either positive or negative effects on insulin signal transduction, depending on the ROS concentration. Our data suggest that the overall concentration of ROS, irrespective of the subcellular compartments in which they act, determines whether they enhance or impair insulin signal transduction.

The observed negative effects of high H_2O_2 concentrations on insulin signal transduction are consistent with our previous report, which showed that mitochondria-derived ROS induce insulin resistance in H4IIEC hepatocytes treated with palmitate by activating JNK [6]. Many lines of evidence suggest that chronic accumulation of ROS has a causal role in the development of insulin resistance [4,5]. The activation of stress kinases such as JNK is thought to play a central role in the development of ROS-associated insulin resistance. The upstream molecules that regulate JNK phosphorylation in response to increases in cellular ROS levels include thioredoxin (TRX) and apoptosis signal-regulating kinase 1 (ASK1) [9,10]. ROS oxidizes TRX and consequently removes it from pre-existing TRX-ASK1 complexes, leading to the activation of ASK1 and JNK signaling. The results of our previous *in vitro* study indicated that using antioxidants to remove ROS and consequently suppress JNK activation has the potential to improve insulin sensitivity [6]. To date, however, the larger clinical intervention trials conducted to evaluate the potential of antioxidant supplements in preventing the development of diabetes have been unable to observe any positive effects [11,12,13]. These conflicting findings led us to hypothesize that the complete removal of ROS from cells does not necessarily improve insulin resistance, and to pay particular attention to the dose-dependent dual actions of ROS on insulin signaling.

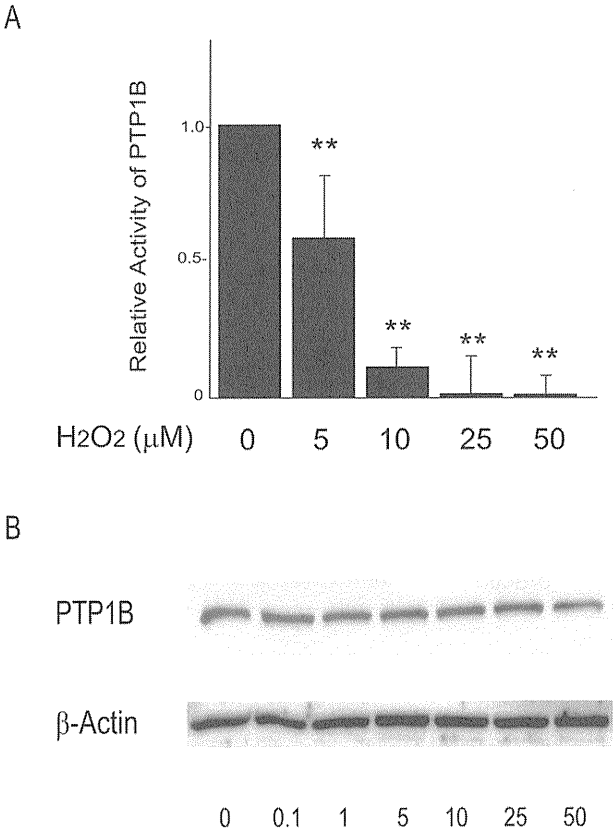

Figure 4. Effects of H2O2 on PTP1B activity in H4IIEC hepatocytes. (A) H4IIEC cells were serum-starved overnight and then treated with the indicated concentrations of H2O2. After snap-freezing in liquid N2, cells were disrupted through scraping into ice-cold RIPA buffer containing a protease inhibitor cocktail, followed by brief sonication and clearing of the resulting lysates by centrifugation at 15,000 rpm for 15 min. PTP1B activity was determined using a CycLex® Protein Tyrosine Phosphatase 1B (PTP1B) Fluorometric Assay Kit. Fluorescence values from three independent experiments were normalized to total protein concentrations and are expressed as mean fold increases over control ± S.D. ** $p<0.01$, versus untreated control. (B) Protein levels of PTP1B were measured by Western blotting.
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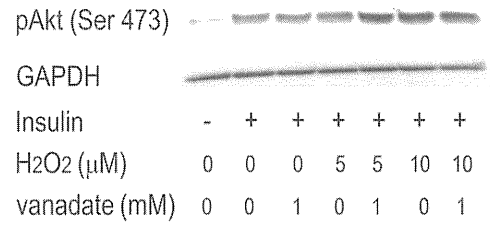

Figure 5. Effects of vanadate on insulin-stimulated Akt phosphorylation in H4IIEC hepatocytes. H4IIEC cells were serum-starved overnight. Then the cells were treated with the indicated concentrations of H_2O_2 for 3 h. Then, the cells were treated with 1mM of sodium orthovanadate for 30 min, and stimulated with insulin (1 ng/mL) for 15 min. Total cell lysates were resolved by SDS-PAGE, transferred to a PVDF membrane, and probed using the indicated antibodies.
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The most surprising finding from our study is that H_2O_2 can exert both positive and negative effects on insulin signaling within a relatively narrow concentration range of 5–50 μM. Banerjee et al. reported blood levels of H_2O_2 to be approximately 1.7±2.5 μM in healthy humans [14], suggesting that our finding of enhanced insulin signaling with 5–10 μM H_2O_2 reflects a physiological, rather than pharmacological, action of H_2O_2. H_2O_2 is reported to have insulin-mimetic actions in insulin target cells [15,16]. However, in these earlier studies, cells were treated with millimolar concentrations of H_2O_2 for short periods of time. To our knowledge, the present study is the first to demonstrate that mild oxidative stress, such as that caused by 5–10 μM H_2O_2, enhances intracellular insulin-mediated signaling. Recent reports have proposed that cells repeatedly exposed to sublethal stresses can develop stress resistance and display increased survival rates, as a result of a process called hormesis. The initial evidence for this novel concept was obtained using model organisms such as nematodes [17] and rats [18]. More recently, Ristow et al. extended the concept of hormesis to humans. They showed that exercise-induced low-grade oxidative stress improves insulin sensitivity in skeletal muscle [19]. However, few reports detailing the dual effects of H_2O_2 on insulin signaling in insulin target cells have been published. The cellular insulin signaling might be maintained by continuous exposure of the cells to low levels of ROS, while insulin resistance develops when cellular H_2O_2 levels rise above a certain threshold.

We show that pretreatment with NAC had a tendency to reduce insulin signaling in both H_2O_2-treated and untreated cells (Fig. 9). In addition, we found that the cells treated with NAC and H_2O_2 showed lower levels of ROS than those in the absence of H_2O_2 (Fig. 8), suggesting that the current concentrations of NAC had a potent anti-oxidative capacity. Goldstein et al. have reported that endogenous H_2O_2 production stimulated by insulin is needed for the subsequent insulin signaling by inactivating phosphatases such as PTP1B [7]. Hence, we speculate that pretreatment with NAC inhibited insulin signaling generally by removing insulin-induced endogenous H_2O_2 production required for the suppression of phosphatase activity due to its strong anti-oxidative capacity.

In our experimental system, it was hard to determine the minimum threshold concentrations of which H_2O_2 exerts promotive effects on insulin signaling, because anti-oxidative capacity in the cells seemed to vary at individual experiments depending on only a modest variation of the viability or numbers of the cells cultured. However, we found that 5–10 μM were the concentrations of which H_2O_2 certainly promotes insulin signal transduction in our cell lines, because these concentrations of H_2O_2 not only increased insulin-stimulated Akt phosphorylation reproducibly (Fig. 2), but also suppressed PTP1B activity significantly (Fig. 4). Thus, we used 5–10 μM of H_2O_2 to certainly increase insulin signaling in the following experiments.

Our findings indicate that the enhancement of insulin signaling by low concentrations of H_2O_2 is accompanied by the suppression of PTP1B activity, and is rescued by the co-administration with vanadate, a non-specific inhibitor for phosphatases. We showed that vanadate further increased insulin signaling in the cells treated with 5 μM of H_2O_2, but not in those treated with 10 μM of H_2O_2 (Fig. 5). These findings seem to be reasonable, because our results showed that 5 μM of H_2O_2 decreased PTP1B activity by 40%, whereas 10 μM of H_2O_2 decreased it by approximately 90% (Fig. 4). Thus, we speculate that co-administration with vanadate was unable to further enhance insulin signaling in the cells where PTP1B activity was almost completely suppressed by 10 μM of H_2O_2. These results suggest that low concentrations of H_2O_2 enhance insulin signaling, at least partly, by inhibiting phosphatases such as PTP1B. PTP1B, a protein tyrosine phosphatase (PTP), negatively regulates insulin signaling by dephosphorylating

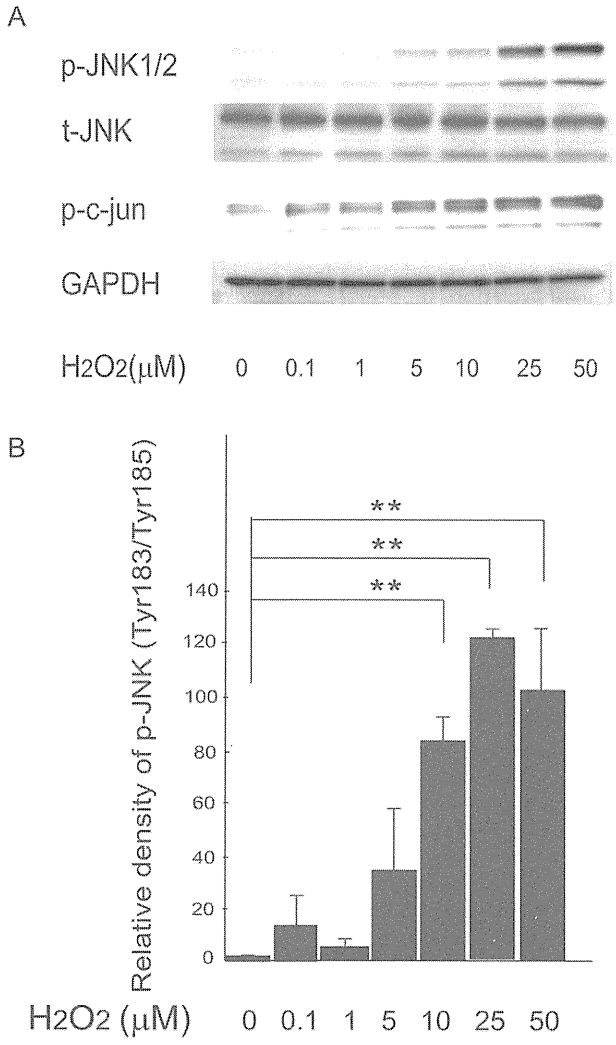

Figure 6. Effects of H2O2 on JNK activation in H4IIEC hepatocytes. (A) H4IIEC cells were serum-starved overnight and then treated with the indicated concentrations of H2O2. Total cell lysates were resolved by SDS-PAGE, transferred to a PVDF membrane, and probed with the indicated antibodies. Signals were detected chemiluminescence. Representative blots are shown. (B) Quantitative data from densitometric analysis of p-JNK signals from three independent experiments were normalized to the values for total JNK and are expressed as mean fold increases over control ± S.D. ** $p<0.01$, versus untreated control.
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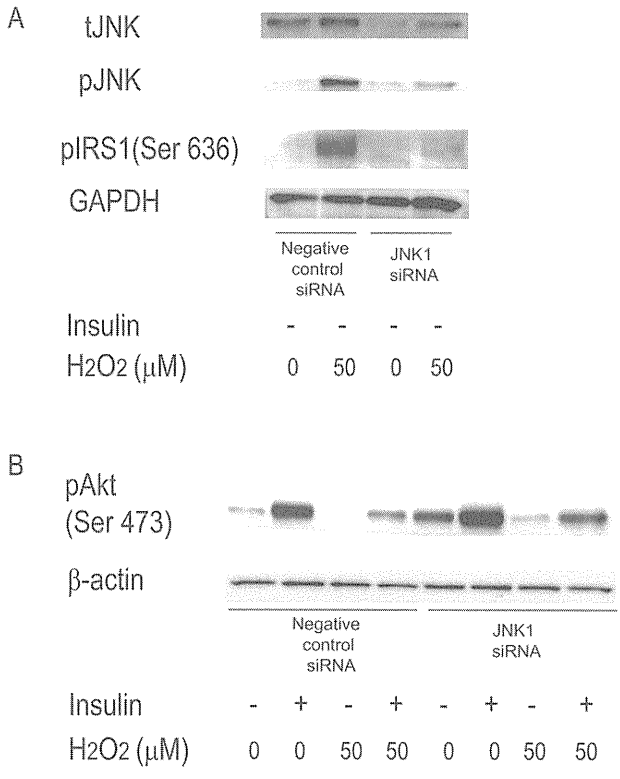

Figure 7. Effects of JNK knockdown on H_2O_2-induced alterations of insulin signal transduction in H4IIEC hepatocytes. H4IIEC cells were transfected with siRNA specific to JNK1 or negative control. 24 h later, the cells were serum-starved overnight. Then the cells were treated with the indicated concentrations of H_2O_2 for 3 h, and stimulated with insulin (1 ng/mL) for 15 min. Total cell lysates were resolved by SDS-PAGE, transferred to a PVDF membrane, and probed using the indicated antibodies. (A) Protein levels of total JNK, phosphorylated INK, and phosphorylated serine residue of IRS-1 in the cells transfected with JNK. (B) Protein levels of phosphorylated Akt in the cells stimulated with insulin.
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a tyrosine residue of the insulin receptor [20]. The structure of the active site and the low pKa of the thiol group of the invariant catalytic cysteine residue render PTP1B highly susceptible to reversible oxidation by H_2O_2 [21]. Oxidation of its active site cysteine residue abolishes PTP1B's nucleophilic properties, causing it to be inactivated. Goldstein et al. showed that an insulin-stimulated endogenous H_2O_2 burst positively regulated insulin signaling, at least partly through oxidative inhibition of PTP1B [7]. However, early papers showed that compounds of vanadium inhibit several forms of phosphatases including PTP1B [22,23]. Thus, additional studies are needed to determine whether low concentrations of H_2O_2 enhance insulin signaling in a PTP1B-specific manner.

Peak IR phosphorylation was observed in the cells treated with the lower concentrations of H_2O_2 compared with Akt phosphorylation (Fig. 2). These results suggest that the action mechanisms of low concentrations of H_2O_2 are different on the phosphorylation of Akt and IR. Several kinds of phosphatases are known to inactivate insulin signaling cascade by dephosphorylating different kinds of downstream effectors of insulin. Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) dephosphorylates phosphatidylinositol 3, 4, 5-trisphosphate (PIP3) to terminate PI3 kinase signaling, resulting in a selective inhibition of PI3 kinase/Akt signaling, although PTP1B dephosphorylates tyrosine residue of insulin receptor, resulting in a total inhibition of insulin signaling. PTEN is also inactivated by H_2O_2–induced oxidation as well as PTP1B [24]. The different sensitivities of H_2O_2 to the phosphorylation of Akt and IR might be derived from the difference in threshold levels of H_2O_2 for the inactivation of various phosphatases, such as PTEN and PTP1B that target Akt and IR, respectively.

Our results identify JNK and phosphatases such as PTP1B as candidate molecules that determine the thresholds for the diametrical effects of H_2O_2 on cellular insulin signaling. We propose that the action of H_2O_2 on cellular insulin signaling is determined by the net balance between inactivation of PTP1B (the

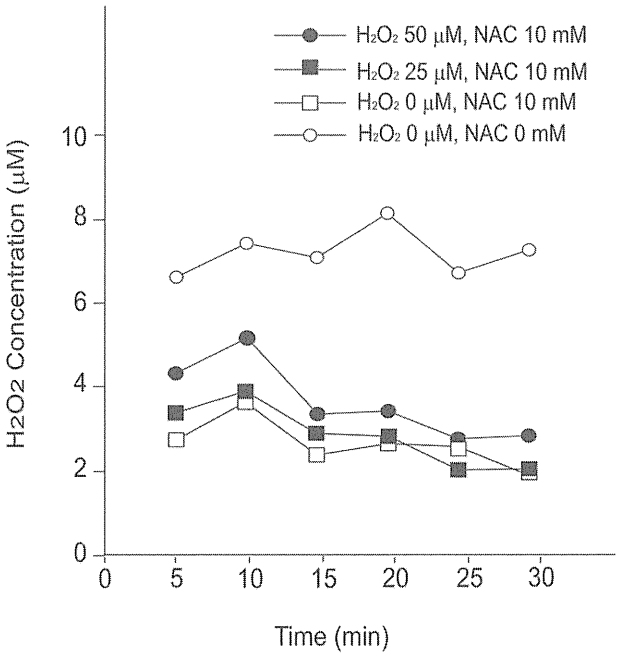

Figure 8. Time course of extracellular H2O2 concentration following its administration to H4IIEC hepatocytes pretreated with *N*-acetyl-L-cysteine. H4IIEC cells were serum-starved and treated with 10 mM of N-acetyl-L-cysteine overnight. Then, the cells were incubated with the indicated concentrations of H2O2 for the indicated periods of time, and the concentration of H2O2 in the culture medium was measured by ferrous oxidation of xylenol orange (FOX) assay.
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enhancive factor for insulin signaling) and activation of JNK (the inhibitory factor for insulin signaling) (Fig. 10). For example, 25–50 μM of H_2O_2 significantly decreased PTP1B activity, whereas these concentrations of H_2O_2 dramatically increased JNK phosphorylation simultaneously. As a result of the summation of these opposite two factors, insulin-induced Akt phosphorylation was impaired by 25–50 μM of H_2O_2 (Figure 2B), suggesting that the increase in JNK activation has a greater influence on net insulin signaling than the inactivation of PTP1B in these concentrations of H_2O_2. On the other hands, 10 μM of H_2O_2 induced a dramatic decrease in PTP1B activity and a moderate increase in JNK phosphorylation, resulting in an enhancement of insulin-stimulated Akt phosphorylation. These results suggest that the decrease in PTP1B activity has a greater influence than the increase in JNK phosphorylation in the cells treated with 10 μM of H_2O_2.

Early *in vitro* studies indicated that in the presence of H_2O_2, the pKa values and reaction rates for PTP1B were lower than those for TRX, an upstream regulator of JNK [25], suggesting that PTP1B may be more susceptible than TRX to oxidation by H_2O_2 *in vivo*. Thus, the dual actions of H_2O_2 on insulin signal transduction in H4IIEC hepatocytes may stem from the difference in susceptibility to H_2O_2 between PTP1B and TRX, although our study leaves open the possibility that other molecules in addition to PTP1B and TRX may participate in the dual effects of H_2O_2. Moreover, because we tested only the membrane-permeable ROS H_2O_2, we cannot exclude the possibility that factors other than concentration, such as intracellular localization, duration, or type of ROS, may influence the action of ROS on insulin signaling. Further investigations are required to elucidate the detailed molecular mechanisms underlying the dual effects of H_2O_2 on insulin signal transduction.

One limitation of our study is that our experiments were performed only in H4IIEC hepatocytes. At present, there are few reports demonstrating the dual action of ROS on insulin signaling in other cell lines. However, Loh et al. showed that mouse embryonic fibroblasts lacking Gpx1, an antioxidative enzyme involved in the removal of H_2O_2, exhibit enhanced insulin-induced Akt phosphorylation and PTEN oxidation, and normal tyrosine phosphorylation of the IR [8]. Thus, the positive regulation of insulin signaling by ROS may not be exclusive to H4IIEC hepatocytes. However, in the current study, we treated differentiated C2C12 myotubes with many concentrations of H_2O_2, but we could not find the dual effects of H_2O_2 on insulin signaling (data not shown). This result suggests that H_2O_2-induced dual effects on insulin signaling might be hepatocytes-specific, but additional studies on other insulin target cells such as adipocytes are needed to confirm that low concentrations of H_2O_2 enhance insulin signal transduction.

In conclusion, the current study demonstrates that depending on its concentration, H_2O_2 can exert either a positive or negative effect on insulin signal transduction in H4IIEC hepatocytes, and suggests that the intracellular ROS concentrations and different susceptibilities of signaling molecules to ROS are major determinants of impaired or enhanced insulin signaling. Further investigations into the dual effects of ROS on insulin signaling may lead to the development of novel antioxidative therapies to selectively combat the oxidative stresses that cause insulin resistance.

Materials and Methods

Materials

Antibodies against phospho-IGF-1 receptor (Tyr1131), phospho-insulin receptor (Tyr1146), insulin receptor β, Akt, phospho-Akt (Ser473), SAPK/JNK, phospho-SAPK/JNK (Thr183/Tyr185), phospho-GSK-3 (Ser21/9), and phosphor-serine636 of IRS-1 were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies against GSK-3 and phospho-c-Jun were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Human recombinant insulin was purchased from Sigma–Aldrich (St. Louis, MO, USA). Hydrogen peroxide, $TiSO_4$, xylenol orange, ammonium ferrous sulfate, and sorbitol were obtained from Wako Chemical Co., Ltd. (Osaka, Japan). PTP1B inhibitor came from Merck (Tokyo, Japan).

The rat hepatoma cell line H4IIEC was purchased from the American Type Culture Collection (Manassas, VA).

Cell Harvesting and Western Blot Analysis

Cells were cultured in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS (Invitrogen), penicillin (100 U/mL), and streptomycin (0.1 mg/mL; Invitrogen) at 37°C in a humidified atmosphere containing 5% CO_2. The cells were cultured to 60–70% confluence in 12-well plates. Next, the cells were serum-starved overnight and then treated with H_2O_2 for a total of 3 h, with medium changes every 30 min. After treatment, they were stimulated with recombinant human insulin (1 ng/mL) for 15 min, washed with ice-cold phosphate-buffered saline, and then lysed in RIPA buffer (Millipore, Billerica, MA) containing a Complete™ Protease Inhibitor Cocktail Tablet, EDTA-free (Roche Diagnostics) and PhosSTOP Phosphatase Inhibitor Cocktail Tablets (Roche Diagnostics). The lysates were sonicated using a Bioruptor sonicator (Cosmo Bio, Tokyo, Japan), and insoluble materials were removed by centrifugation. The resulting

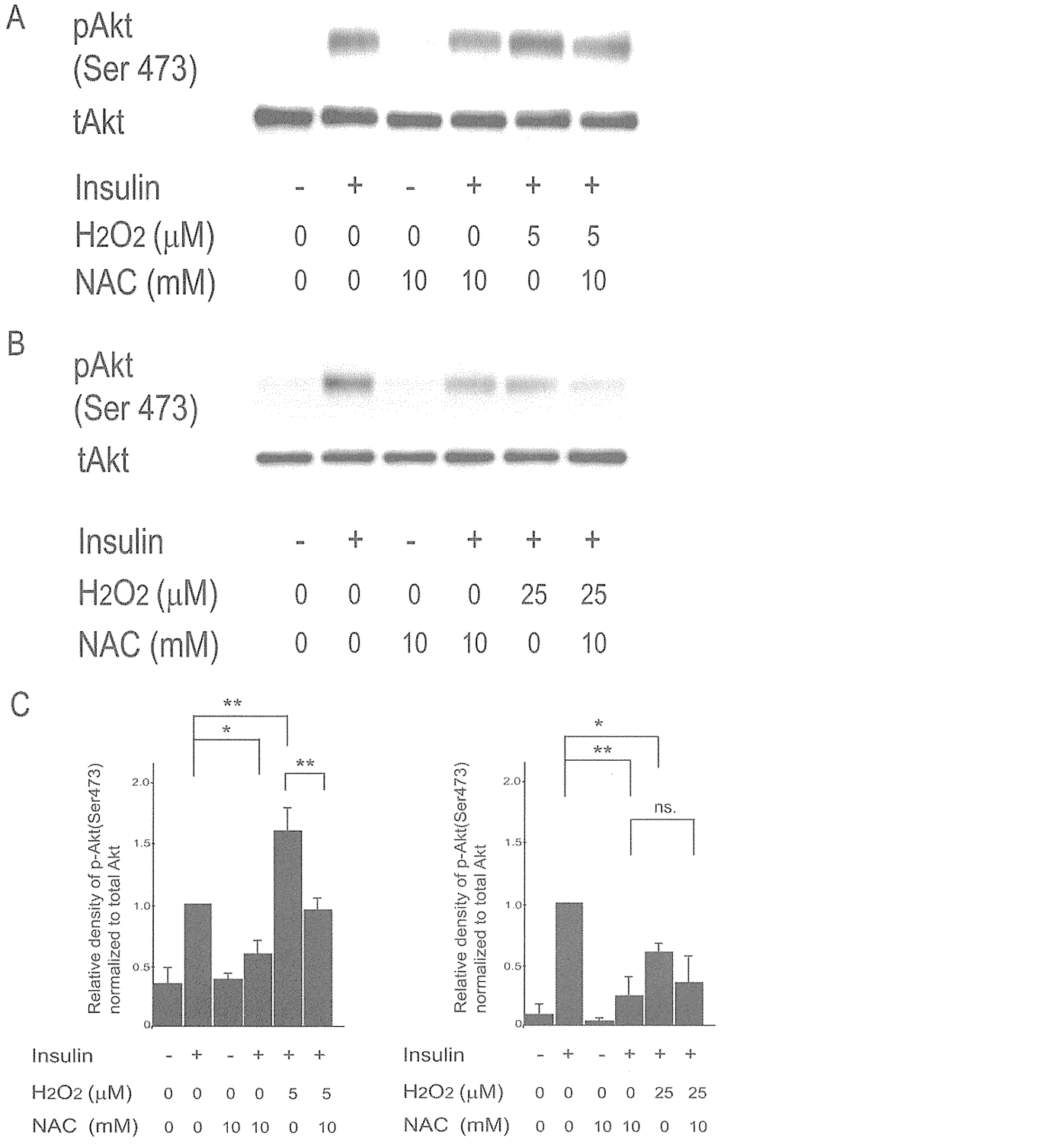

Figure 9. Effects of antioxidant *N*-acetyl-L-cysteine on H_2O_2-induced alterations of insulin signal transduction in H4IIEC hepatocytes. (A–B) H4IIEC cells were serum-starved and treated with 10 mM of N-acetyl-L-cysteine overnight. Then the cells were treated with the indicated concentrations of H_2O_2 for 3 h, and stimulated with insulin (1 ng/mL) for 15 min. Total cell lysates were resolved by SDS-PAGE, transferred to a PVDF membrane, and probed using the indicated antibodies. (C) Quantitative data from densitometric analysis of p-Akt signals from three independent experiments were normalized to the values for total Akt and are expressed as mean fold increases over control ± S.E.M. . * $p<0.05$, versus treatment with insulin alone; ** $p<0.01$, versus treatment with insulin alone.
doi:10.1371/journal.pone.0027401.g009

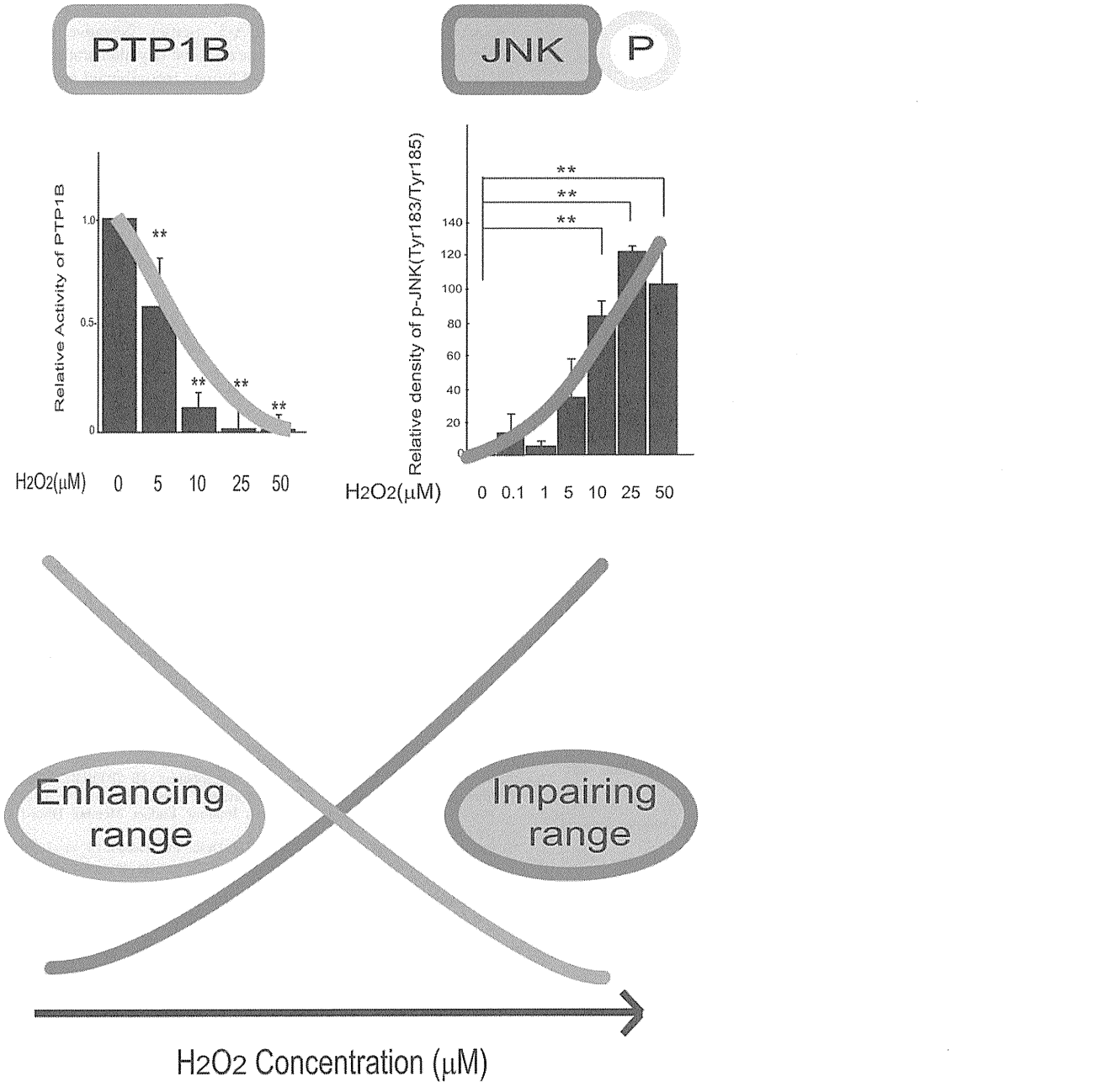

Figure 10. Dual action of H2O2 on insulin signal transduction can be explained by different thresholds for the repression of PTP1B activity and enhancement of JNK activation.
doi:10.1371/journal.pone.0027401.g010

supernatants were separated by SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore). The membranes were blocked in a buffer containing 5% nonfat milk, 50 mM Tris (pH 7.6), 150 mM NaCl, and 0.1% Tween 20 for 1 h at room temperature and then incubated with specific primary antibody, followed by horseradish peroxidase-linked secondary antibody. Signals were detected using a chemiluminescence detection system (ECL Plus Western blotting detection reagent; GE Healthcare Bio-Sciences, Piscataway, NJ, USA). The membranes were subjected to direct densitometric analysis using a CCD camera system (LAS-3000 mini; Fuji-film, Tokyo, Japan) and Scion Image software.

Measurement of H_2O_2 Concentrations in Culture Medium

Hydrogen peroxide concentrations in the culture medium were measured by ferrous oxidation of xylenol orange (FOX) assay [26]. Samples of culture media were added at specific intervals to FOX reagent, which comprised 100 μM xylenol orange, 250 μM ammonium ferrous sulfate, 100 mM sorbitol, and 25 mM H_2SO_4. Changes in absorbance at 560 nm were measured, and

concentrations of H_2O_2 were calculated using a standard curve generated using H_2O_2 solutions of known concentrations.

Preparation of H_2O_2

Hydrogen peroxide (30% v/v; Wako Chemical. Co., Ltd.) was diluted to a concentration of 100 μM in distilled water. The precise concentration of hydrogen peroxide was determined using the titanium oxide method [27], in which the molar coefficient of a titanium oxide-hydrogen peroxide complex is assumed to be 750 M^{-1} cm^{-1} at 405 nm. Briefly, 160 μl of hydrogen peroxide solution (prepared as described above) were added to a mixture of 30 μl of titanium sulfate and 50 μl of 20% (v/v) hydrogen sulfate. The resulting mixture was stirred at room temperature for 15 min, and the precise concentration of hydrogen peroxide was calculated from the absorbance at 405 nm.

siRNA transfection in H4IIEC hepatocytes. H4IIEC hepatocytes were transiently transfected with 30 nM of siRNA duplex oligonucleotides using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions, as described previously [28]. A JNK1-specific siRNA was synthesized by Dharmacon (USA): 5′-GAAUAGUGUGUGCAGCUUA-3′ (sense). Negative control siRNA was purchased from Dharmacon. One day after transfection, cells were stimulated with 1 ng/ml of human recombinant insulin for 15 min.

Statistical Analysis

Differences between two groups were tested by unpaired, two-tailed Student's *t*-tests. More than two groups were compared by one-way analysis of variance (ANOVA). All calculations were performed using StatView software (SAS Institute Inc., Cary, NC, USA).

Author Contributions

Conceived and designed the experiments: SI HM SM SK TT. Performed the experiments: SI MS TT. Analyzed the data: SI HM. Contributed reagents/materials/analysis tools: SI HM MS SM. Wrote the paper: SI HM.

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糖尿病性腎症における脂質代謝異常とその役割

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abstract

透析導入原疾患の第一位である糖尿病性腎症は，厳格な血糖・血圧のコントロールおよびレニン・アンジオテンシン（renin-angiotensin：RA）系阻害薬の有効性が確立されているにもかかわらず，増加の一途をたどっている．近年では脂質代謝異常をはじめとする種々の生活習慣病の合併によりその病態は複雑となってきている．実際，疫学調査の結果から脂質代謝異常は糖尿病性腎症の発症・進展の独立した危険因子であることが明らかとなっており，その重要性が再認識されている．慢性腎臓病（chronic kidney disease：CKD）および糖尿病ではカイロミクロン（chylomicron：CM）やVLDL，IDLといったレムナントが増加する．これらはtriglyceride-rich lipoprotein（TGRL）とよばれ，糖尿病性腎症の病態に深く関与する．脂質異常症治療薬によって糖尿病性腎症の進展が抑制されることも報告されており，血糖や血圧に加えて脂質管理を行うことでさらなる腎予後の改善につながるものと期待される．

I　はじめに

糖尿病性腎症は1998年以降，透析導入原疾患の第一位となり，以後もその割合は増加の一途をたどっている．2009年には16,414名（全体の44.5%）が糖尿病性腎症から透析導入となっており[1]，この絶対数の抑制は社会的課題ともいえる．糖尿病性腎症の治療にはHbA1c値を6.5%未満とする厳格な血糖コントロール[2]，ならびに主にレニン・アンジオテンシン（renin-angiotensin：RA）系阻害薬を第一選択とした130/80mmHg未満（尿タンパク 1g/日以上の症例では125/75mmHg未満）を目標とする厳格な血圧コントロール[3]が必要不可欠である．さらに血糖・血圧のみならずスタチンによる脂質管理〔血清総コレステロール＜175mg/dL，血清トリグリセリド（TG）＜150mg/dL〕やアスピリン，抗酸化薬の内服などを合わせた集約的治療により大血管障害のみならず腎症の進展も有意に抑制され，5年間の追跡調査で末期腎不全への進行が有意に抑制された[4]ことが示されている．わが国においても，Arakiらは微量アルブミン尿を呈する2型糖尿病患者を対象とした6年間の追跡調査により血糖・血圧・脂質それぞれの目標達成項目数が多いほどアルブミン尿の寛解率が高いことを確認している[5]．これらの結果は脂質管理あるいは脂質代謝異常治療薬そのものの腎保護効果を示唆するものと考えられる．本稿では糖尿病性腎症における脂質代謝異常の疫学およびその特徴，腎症悪化の分子機序，そして今後の治療展開について触れる．

II　糖尿病性腎症悪化因子としての脂質代謝異常

糖尿病性腎症の治療において血糖コントロールが

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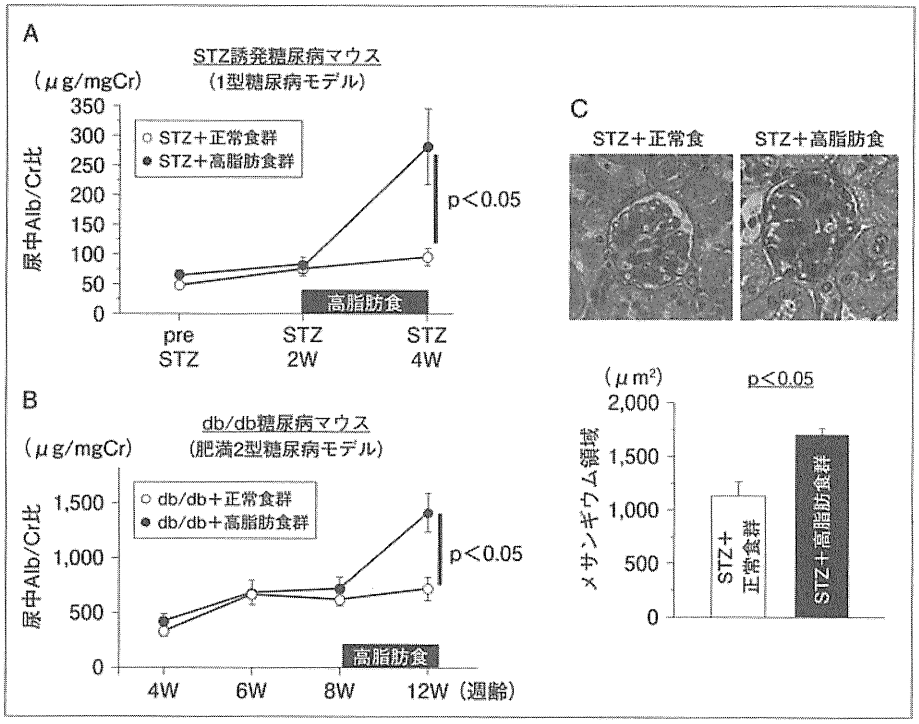

図1
糖尿病モデルマウスへの高脂肪食負荷による検討
A，B：1型および2型糖尿病マウスに高脂肪食負荷を行った時のアルブミン尿の経時的推移．C：PAS染色によるメサンギウム領域の評価とその定量．
STZ：ストレプトゾトシン

最重要なのは当然であるが[6]，実際には1型糖尿病患者全体の約1/3しか腎症を発症しないことから，ジェネティックあるいはエピジェネティック，環境因子，他の代謝的側面が重要であることは明らかである．数々の疫学調査によって，脂質代謝異常が大血管障害のみならず糖尿病性腎症発症・進展の独立した危険因子であることが報告されている．初発の2型糖尿病患者5,102名を15年間観察したUKPDSでは観察開始時の高TG血症，低HDL血症はそれぞれアルブミン尿発症，血清クレアチニン倍化と相関していたことが報告されている[7]．また，2型糖尿病患者1,513名を対象としたRENAAL studyのサブ解析においても観察開始時の総コレステロール，LDLコレステロールの高値は末期腎不全到達のリスクであることが報告されている[8]．

Ⅲ 糖尿病性腎症で認める脂質代謝異常の特徴

糖尿病性腎症では糖尿病，腎機能低下，ネフローゼ症候群といった種々の要素が絡んで脂質代謝異常を合併し得る．糖尿病ではインスリン作用不足によってリポタンパクリパーゼ（lipoprotein lipase：LPL）活性が低下する．また慢性腎不全ではLPLおよび肝性トリグリセリドリパーゼ（hepatic triglyceride lipase：HTGL）の活性低下を認める．これらの活性不足によってカイロミクロン（chylomicron：CM），VLDLあるいはIDLの異化が障害され，CM，VLDL，レムナントを含むtriglyceride-rich lipoprotein（TGRL）の増加，高IDL血症，低HDL血症を認める．ネフローゼ症候群では低タンパク血症を代償するために肝臓でのタンパク合成亢進が起こり，VLDL産生が増加する．強度のネフローゼではアポC-Ⅱの尿中への漏出が増加することでLPLの作用不足が引き起こされ，VLDLの増加が増強する．TGRLを主体とするこれらnon-HDLコレステロールは動脈硬化促進的に働き，腎病変の進展にも積極的な役割を果たすと考えられている[9]．このほか，2型糖尿病性腎症患者ではレムナントやVLDLの構成要素であるアポEのe2アレルの保有率が高いことが報告されている[10]ことから，e2アレルで高頻度に認められるⅢ型高脂血症や高レムナント血症の関与が示唆される．

Ⅳ 脂質代謝異常が糖尿病性腎症を悪化させる分子機序

前述の疫学調査の結果から糖尿病性腎症の独立した危険因子として脂質代謝異常が働く可能性が示唆された．また，われわれは1型および2型糖尿病モデルマウスに対する高脂肪食負荷が腎症を明らかに悪化させることを確認している（図1）．悪化機序には非常に多因子の関与が想定されているが，今回，主なものについて概説する．

1 TGF-βシグナル経路

transforming growth factor（TGF）-βは病態のなかで最も重要な役割を果たしているもののひとつであり，糸球体ではメサンギウム基質拡大や結節性病変を，また間質の線維化を促進するkey moleculeとして知られている．本シグナル経路の活性化は高血糖刺激，酸化ストレス，終末糖化産物（advanced glycation end-products：AGEs），RA系活性化などによって誘導されるため，後述の複数の因子と絡んでvicious cycleを形成すると考えられる．TGRLの代謝産物や酸化LDLはメサンギウム細胞[11]や内皮細胞[12]に対して直接あるいは活性酸素種（reactive oxygen species：ROS）産生を介してTGF-β産生を刺激し糸球体硬化を促進する．

2 レニン・アンジオテンシン系

糖尿・肥満状態におけるRA系の亢進が腎障害に深く関与し，RA系阻害薬が病態の改善に有効であることは臨床的にも広く知られている．これまでのわれわれの検討のなかで，糖尿病モデルマウスに対する高脂肪食負荷が腎組織RA系を強く亢進させることがわかっている（図2）．この腎局所で活性化したRA系はROSの産生亢進，LDLやレムナントの酸化促進，TGF-β経路の活性化などを介して腎障害を引き起こす．われわれは糖尿病・高脂血症合併モデルマウスに非降圧量のアンジオテンシンⅡ受容体拮抗薬（ARB）投与を行い，脂質負荷により引き起こされるRA系活性化，腎症進展に対してもARBが有効である可能性を見いだしている．

3 酸化ストレス

糖尿病で増加する異常脂質は糸球体でのROS産生を亢進することで酸化ストレスを惹起する．他，

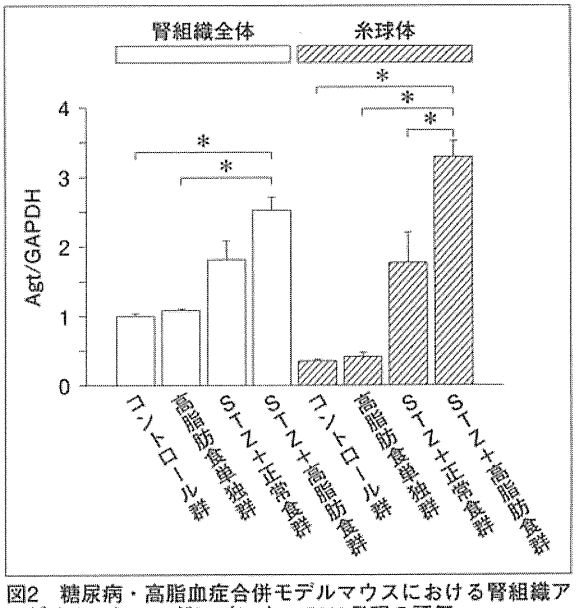

図2 糖尿病・高脂血症合併モデルマウスにおける腎組織アンジオテンシノーゲン（Agt）mRNA発現の評価
定量的PCRによる評価．STZ＋正常食群で腎組織Agt発現が増加，STZ＋高脂肪食群ではさらに強い発現増加を認めた．＊：$p<0.01$

糖尿病性腎症患者で増加を認めるカイロミクロンレムナントは血管壁を透過しやすいことから動脈硬化促進的に作用しやすく[13]，またLDLやVLDLなどは高血糖状態で糖化酵素非依存的に糖化修飾を受け，この糖化リポタンパクは酸化されやすいため糖尿病において腎症をはじめとする血管合併症リスクの一因ともなる[14]．これらのことより，糖尿病性腎症患者に脂質代謝異常を合併した状態はきわめて酸化ストレスを亢進させやすい環境と思われる．

4 内皮障害

糖尿病性腎症において糸球体上皮細胞，メサンギウム細胞の障害が重要であることはよく知られているが，最近になり内皮細胞障害も一定の役割を果たしていることがわかってきた．内皮細胞の表面にはglycocalyxという微小構造が存在しており，糸球体ではタンパク透過性を制限するバリアーとして働いていると考えられている．Kashiharaらは糖尿病モデルラットを用いた検討でアルブミン尿の発症にROSによる内皮障害，そしてglycocalyxの劣化が重要であることを報告している[15]．糖尿病性腎症で増加するTGRLは直接，あるいはROSの産生，炎症などを介して内皮障害，glycocalyxの劣化を引き起こすとされる[9]．

5　炎症・マクロファージ

炎症は動脈硬化の成因[16]として広く認識されている．糖尿病性腎症は細血管障害であり，大血管障害である動脈硬化とは病変の主座となる血管が異なるものの，マクロファージ（macrophage：Mφ）浸潤，炎症に関連するサイトカイン発現増加など，共通した変化も認められる．実際，糖尿病性腎症の腎組織においてMφ浸潤が認められ[17]，Mφに発現しているスカベンジャー受容体を欠損させることで炎症およびMφ浸潤が抑制されると同時に糖尿病性腎症が軽減する[18]ことから，動脈硬化と同様に糖尿病性腎症においても炎症・Mφは一定の役割を果たしていると思われる．現在，われわれは糖尿病モデルマウス糸球体のcDNA microarrayによる網羅的解析を行い病態修飾候補分子を抽出，炎症に関連する受容体とその内因性リガンドに着目して解析を進めている．糖尿病性腎症糸球体で発現増加する両分子は脂質負荷によってさらに発現が増強しており，本シグナル経路の遮断が脂質負荷による腎症悪化の抑制に有効である可能性を確認している．また*in vitro*でもMφに対する高糖濃度・脂質刺激が炎症メディエーターに対して相乗効果を示す結果が得られており，脂質が糖尿病性腎症を悪化させる機序としての炎症・Mφ活性化の役割が注目される．

V　今後の治療展開

以上の疫学調査や基礎的検討の結果から，糖尿病診療において血糖・血圧に加えて脂質を管理することは動脈硬化・大血管障害のみならず腎症の発症・進展抑制にも重要と考えられる．腎保護に有効な可能性のある脂質代謝異常治療薬として，現在スタチン[19]やフィブラート[20]が臨床現場で用いられている．詳細は他稿に譲るが，これらの薬剤は脂質低下作用はもちろんのこと，それ以外の多面的効果によっても臓器保護的に働くことが示されている．エビデンスといえるレベルではないものの脂質管理のためにこれらの薬剤を用いることは有用であると考えられる．脂質を量のみならず“質”の面からも改善させる新規薬剤としてコレステロールエステル転送タンパク（cholesterol ester transfer protein：CETP）阻害薬[21]も腎保護の面から期待される．抗酸化分子を発現制御する重要な転写因子NF-E2-related factor 2（Nrf2）の活性化は糖尿病性腎症を軽減[22]する．Nrf2活性化薬はすでにphase Ⅱbまで治験が行われており，臨床応用可能な新規薬剤として有望視されている．これらのことから脂質を量と質の両面から治療すること，そして脂質による悪化機序において重要な役割を果たすと考えられる酸化ストレスや炎症とそのメディエーターを治療ターゲットとした新規治療薬が開発されることにより，糖尿病性腎症の腎予後が一層改善されることが期待される．

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