Statistical analysis

The statistical analysis of the results was performed by the unpaired Student's *t* test. *P*-values <0.05 were considered significant.

Results

Effects of C-peptide on urinary albumin excretion in streptozotocin-induced diabetic mice

In order to examine the effect of C-peptide on the development of early diabetic glomerulopathy, we made C57Bl/6 mice diabetic by peritoneal injection of STZ, and then administered with either rat C-peptide or scrambled C-peptide. Table 1 shows the characteristics of experimental animals treated with C-peptide, scrambled C-peptide, or saline for 24 h. STZ-diabetic mice had almost twice as high fasting plasma glucose as compared to non-diabetic mice. Diabetic animals showed lower body weight (−13.4%) than the non-diabetic controls. Urine volume and creatinine clearance were significantly increased in the diabetic mice compared with non-diabetic control mice. These values were similar for both C-peptide-treated and scrambled C-peptide-treated mice. Blood pressure was not different among the four groups. Plasma concentration of C-peptide was within physiological range (4.1 ± 1.1 nmol/L) in STZ-diabetic mice administered with C-peptide. C-peptide could not be detected either in non-diabetic or STZ-diabetic mice without C-peptide treatment, since the ELISA system specifically reacted with rat C-peptide and not with that of mouse origin.

As shown in Figure 1, C-peptide administration for 24 h reduced urinary albumin excretion from 0.29 ± 0.03 to 0.18 ± 0.02 μg/min (−40.7% of diabetes-induced increase, $P < 0.01$). There was no significant difference between diabetic mice receiving saline and diabetic mice treated with scrambled C-peptide.

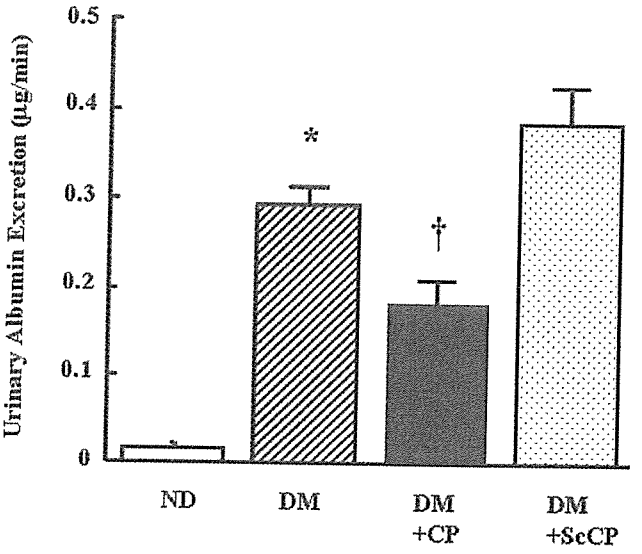

Figure 1. Effect of C-peptide on urinary albumin excretion in diabetic mice. Streptozotocin-induced diabetic mice were intraperitoneally administered with C-peptide or scrambled C-peptide for 24 h. Non-diabetic and diabetic control mice received saline treatment. Urinary albumin excretion was measured by ELISA. Results are given as means ± SEM ($n = 7$). ND, non-diabetic mice; DM, diabetic mice; DM + CP, diabetic mice treated with C-peptide; DM + ScCP, diabetic mice treated with scrambled C-peptide. *$P < 0.01$ vs ND; †$P < 0.01$ *vs* DM

Effects of C-peptide on the expression of TGF-β and type IV collagen in diabetic mice glomeruli

Others and we have previously demonstrated that albuminuria is associated with up-regulation of TGF-β and type IV collagen in STZ-induced diabetic mice glomeruli [24,25]. Therefore, we next examined the effects of C-peptide on mRNA expression of (α3)IV collagen, a major component of GBM, and TGF-β in the isolated glomeruli using real-time PCR. When the animals were

Table 1. Characteristics of experimental groups of mice

	Non-diabetic control ($n = 7$)	STZ-diabetic mice		
		Saline ($n = 7$)	C-peptide ($n = 7$)	Scrambled C-peptide ($n = 7$)
Plasma glucose (mmol/L)	6.7 ± 1.7	13.4 ± 2.8[a]	15.4 ± 3.2[a]	15.8 ± 3.6[a]
Body weight (g)	26.2 ± 0.9	22.7 ± 2.9[b]	20.3 ± 3.5[a]	22.1 ± 3.0[a]
Urine volume (mL/12 h)	1.0 ± 0.7	14.8 ± 2.6[a]	12.0 ± 4.5[a]	15.6 ± 3.1[a]
Blood pressure (mmHg)				
Systolic	94 ± 4.3	88 ± 7.9	90.7 ± 10.9	88.5 ± 9.5
Diastolic	62.8 ± 8.9	53 ± 7.2	58.2 ± 10.3	50.7 ± 8.9
Mean	47 ± 12.5	36.3 ± 10.1	42 ± 12.2	31.7 ± 9.5
Creatinine clearance (mL/hr)	6.09 ± 7.6	38.9 ± 12.9[b]	34.1 ± 8.0[a]	49.5 ± 8.2[a]

Data are means ± SD.
[a]$P < 0.01$.
[b]$P < 0.05$ for difference between STZ-diabetic mice and non-diabetic mice. None of the variables differed significantly between the three diabetic groups.

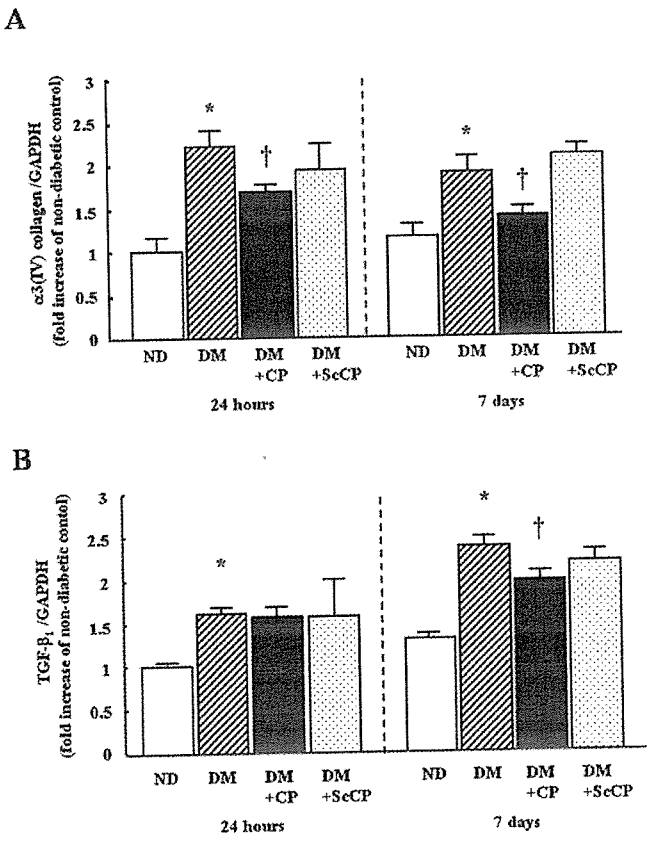

Figure 2. Effect of C-peptide on glomerular mRNA expression of α3(IV) collagen and TGF-β1. Streptozotocin-induced diabetic mice were intraperitoneally administered with C-peptide or scrambled C-peptide, for either 24 h or 7 days. Glomeruli were then isolated by the magnet microbeads method and the total RNA was extracted. Transcript levels of α3(IV) collagen (A) and TGF-β1 (B) were evaluated by quantitative real-time RT-PCR. Results are given as means ± SEM ($n = 7$). *$P < 0.01$ *vs* ND; †$P < 0.05$ *vs* DM

studied after 24 h of C-peptide infusion, the diabetes-induced up-regulation of (α3)IV collagen (2.2-fold) was reduced by 44.6% by C-peptide ($P < 0.05$, Figure 2(A)) in the diabetic mice. Likewise, after 7 days of C-peptide infusion, there was a 68.4% inhibition of the diabetes-induced increase in transcript level of (α3)IV collagen mRNA level ($P < 0.05$). Control studies using scrambled C-peptide showed no effect (Figure 2(A)). In contrast, the expression of TGF-β in glomeruli, up-regulated 1.6-fold in diabetic mice, was not significantly changed by C-peptide treatment for 24 h (Figure 2(B)). However, C-peptide administration for 7 days resulted in significant suppression of TGF-β (−36.6% of diabetes-induced increase, $P < 0.05$).

Effects of C-peptide on TGF-β-induced expression of type IV collagen in cultured mice podocytes

Podocytes play an important role in selective glomerular filtration. They are the main producers of (α3)IV collagen in glomeruli, and their dysfunction is implicated in the development of diabetic nephropathy [26–28]. It has been reported that TGF-β up-regulates type IV collagen expression in podocytes [29]. We therefore examined next whether C-peptide affects the expression of (α3)IV collagen in a podocyte cell line induced by TGF-β *in vitro*. As shown in Figure 3(A), 0.1 ng/mL TGF-β up-regulated the transcript level of (α3)IV collagen more than 3-fold, and treatment with C-peptide (0.1, 0.5, and 1 nM) dose-dependently inhibited the elevation by maximally 84.6% ($P < 0.05$). Western blot analysis confirmed that C-peptide dose-dependently inhibited TGF-β-induced up-regulation of type IV collagen also at the protein level (Figure 3(B)).

The effect of C-peptide on expression of plasminogen activator inhibitor (PAI)-I, a gene well characterized for its responsiveness to TGF-β [30,31], was also examined in podocytes. As shown in Figure 3(C), TGF-β treatment up-regulated PAI-I mRNA expression 2.4-fold, and C-peptide pretreatment suppressed the change by 53.1%.

Effect of PTX and MAPK inhibitors on suppression of type IV collagen expression by C-peptide

An increasing body of evidence suggests a role of G-protein coupling receptors and various MAPKs in the biological functions of C-peptide [11,14,15,17,32–35]. As shown in Figure 4(A), preincubation of podocytes with PTX, a specific inhibitor of $G_{i/o}$ proteins, dose-dependently reversed the suppressive effect of C-peptide on (α3)IV collagen expression. PTX alone in the absence of C-peptide did not affect the transcript level of type IV collagen.

In order to further clarify the signaling pathways involved in the effect of C-peptide in murine podocytes, we next examined whether MAPK inhibitors affected C-peptide function. As shown in Figure 4(B), pretreatment of podocytes with PD98059, a specific inhibitor of MAPK/ERK kinase 1 (MEK1), dose-dependently reversed the suppressive effect of C-peptide on type IV collagen. In contrast, neither SP600125, a SAPK/JNK inhibitor nor SB203580, a p38 MAPK inhibitor had any detectable effect on the expression of type IV collagen (Figure 4(C) and (D)).

Effect of C-peptide on MAPK activities in mice podocytes

We finally tested whether C-peptide activates the three members of the MAPK family, ERK, SAPK/JNK, and p38, in the cultured podocytes. After stimulation, ERK and p38 were collected by immunoprecipitation and SAPK/JNK was collected by adsorption to the c-Jun fusion protein, and were subjected to an *in vitro* kinase assay in the presence of the respective substrates, Elk-1 for ERK, c-Jun for SAPK/JNK, and ATF-2 for p38. Phosphorylation of the substrates was detected by immunoblotting with the specific antibodies against the phosphorylated substrates.

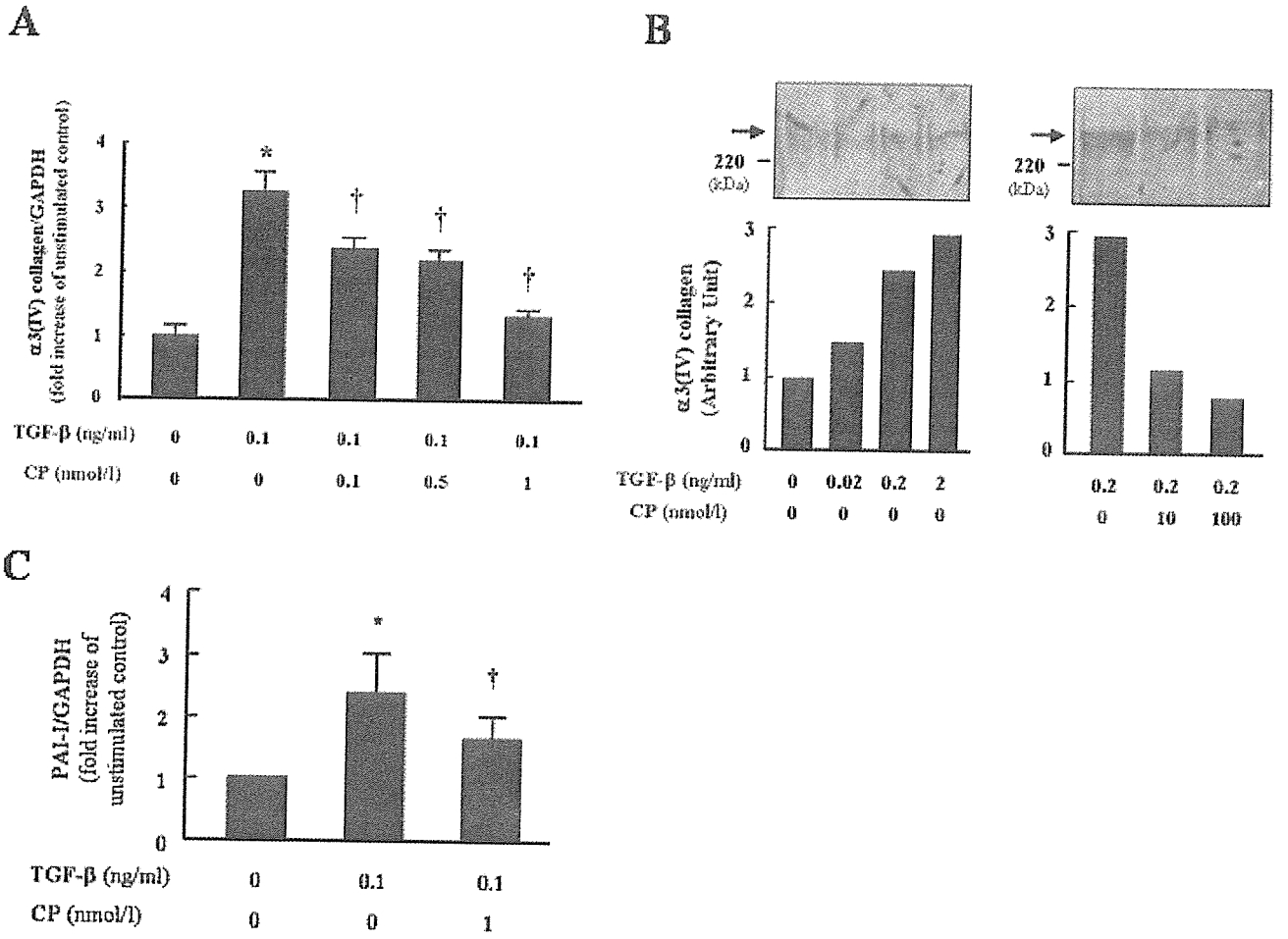

Figure 3. Effect of C-peptide on TGF-β-induced gene expression in cultured murine podocytes. Podocytes preincubated with indicated doses of C-peptide for 30 min were treated with TGF-β for 24 h, and the transcript levels of α3(IV) collagen (A) and PAI-I (C) were evaluated by real-time RT-PCR. Cell lysates were also subjected to western blotting to evaluate protein expression of type IV collagen (B, upper panels), and the type IV collagen bands were quantified by densitometry (B, lower panels). Bars represent means ± SEM ($n = 3$). *$P < 0.01$ *vs* cells without TGF-β treatment. †$P < 0.05$ *vs* cells treated with 0.1 ng/mL TGF-β in the absence of C-peptide. CP, C-peptide. Arrows indicate the migratory positions of type IV collagen

As shown in Figure 5(A) and (B), C-peptide significantly increased ERK activity in podocytes by 6.64 ± 2.0-fold at the dose of 1 nM compared with the baseline, whereas scrambled C-peptide showed no effect on ERK activity. In contrast, activities of p38 and JNK were not affected by C-peptide (Figure 5(A), (C), and (D)).

Discussion

In the present study, rat C-peptide at a physiological concentration significantly reduced urinary albumin excretion and glomerular expression of α3(IV) collagen, a major component of GBM, in streptozotocin-induced diabetic mice. C-peptide given for 7 days also suppressed the TGF-β expression in diabetic mice glomeruli. *In vitro*, C-peptide attenuated TGF-β-induced expression of type IV collagen and PAI-I in cultured murine podocytes. Furthermore, the suppressive effect of C-peptide on TGF-β-induced (α3)IV collagen expression was shown to be mediated by a G-protein coupling receptor and ERK.

Diabetic nephropathy is histopathologically characterized by GBM thickening and mesangial matrix expansion [20]. It is reported that, in STZ-induced diabetic mice model, glomerular hypertrophy and GBM thickening occur in 4–5 weeks, and subsequently, mesangial expansion and sclerosis develop within 20–30 weeks [24,36]. It has been well described that type IV collagen, produced by podocytes and mesangial cells, is a major contributor of such morphological changes [20,37,38]. Although the cause of type IV collagen accumulation has not been fully elucidated, recent findings underscore the role of TGF-β, a potent inducer of extracellular matrix known to be up-regulated in diabetic glomeruli [20,39–42]. And, in fact, TGF-β has been shown to stimulate the expression of type IV collagen in both podocytes and mesangial cells [20,29,43,44]. In this study, we showed for the first time *in vivo* that C-peptide suppresses the transcript level of (α3)IV collagen, a major GBM component, in diabetic glomeruli. C-peptide treatment for 7 days significantly attenuated glomerular expression of (α3)IV collagen as well as TGF-β, implying that C-peptide regulates collagen expression via suppression of TGF-β. On the other

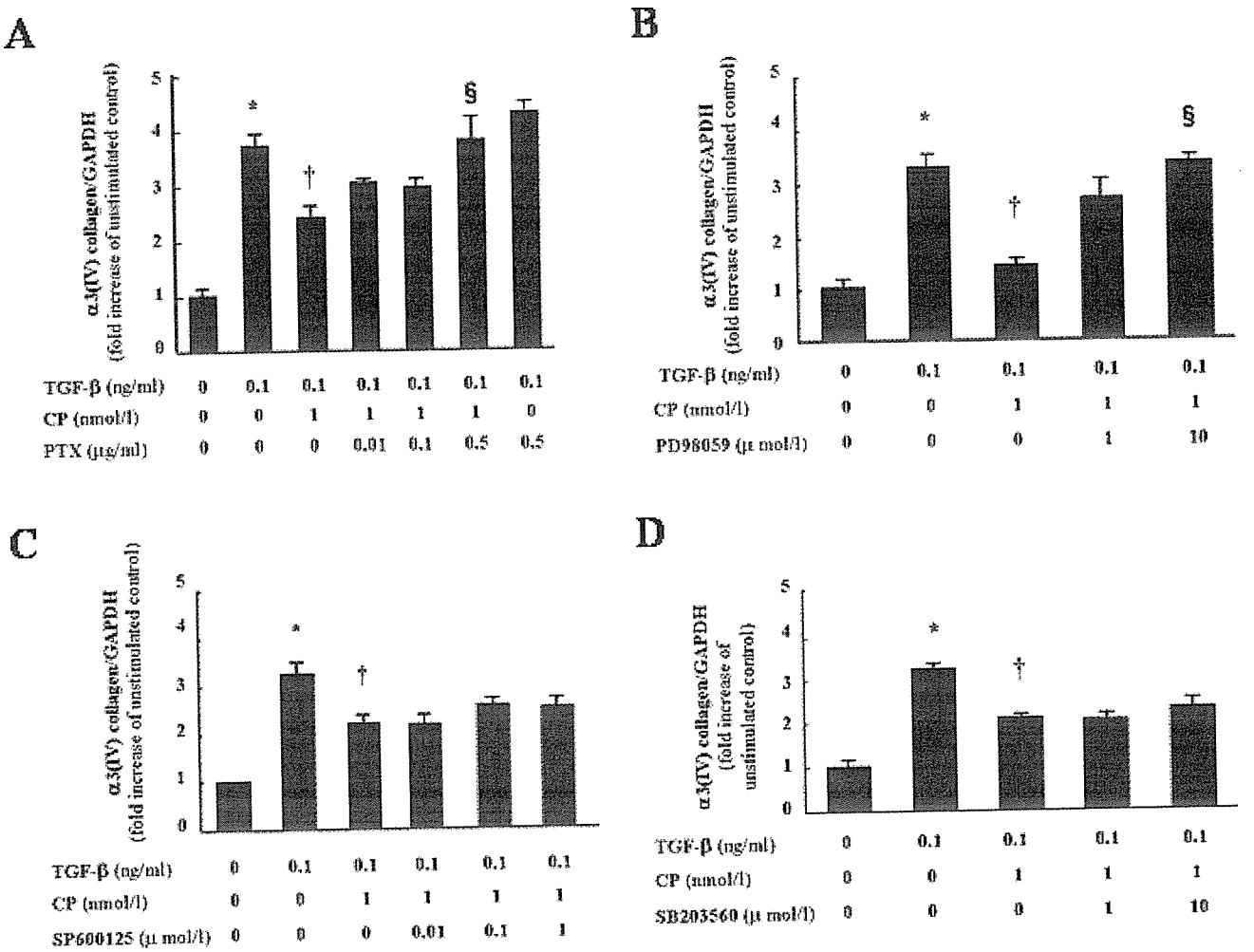

Figure 4. Effect of PTX and MAPK inhibitors on α3(IV) collagen expression modulated by C-peptide. Podocytes were preincubated either with PTX (A), PD98059 (B), SP600125 (C) or SB203560 (D), treated with C-peptide for 30 min, and then with TGF-β for 24 h. The transcript levels of α3(IV) collagen was evaluated by real-time RT-PCR. Results are given as means ± SEM ($n = 3$). *$P < 0.01$ *vs* the cells without TGF-β treatment. †$P < 0.01$ *vs* the cells treated with 0.1 ng/mL TGF-β alone. §$P < 0.01$ *vs* the cells treated with both 0.1ng/mL TGF-β and 1 nmol/L C-peptide. PTX, pertussis toxin

hand, C-peptide administration for a shorter period (24 h) reduced expression of (α3)IV collagen without affecting the amount of TGF-β. This finding raises the possibility that C-peptide suppresses (α3)IV collagen expression not only via inhibition of TGF-β level itself, but also through interference with the signaling cascade downstream of TGF-β.

Our *in vitro* findings indeed support the notion that C-peptide inhibits the effect of TGF-β. We demonstrated that C-peptide suppressed TGF-β-induced type IV collagen expression both at mRNA and protein levels in cultured murine podocytes, the cells that play a major role in production of glomerular (α3)IV collagen. Moreover, C-peptide also suppressed the level of PAI-I, a gene well characterized to be regulated by Smad pathway downstream of TGF-β [30,31]. These results may point to a possible interaction between C-peptide and TGF-β-Smad signaling.

It has been reported that C-peptide in nanomolar concentrations binds specifically to cell membranes, possibly to a G-protein coupling receptor [11]. Intracellular signaling by C-peptide seems to involve the elevation of Ca^{2+} level as well as MAPK activation, leading to up-regulation of Na,K-ATPase [10–15] and endothelial NO synthase [16,17]. In line with these observations, we showed that PTX, a blocker of a G-protein, and PD98059, a specific inhibitor of ERK pathway, removed the suppressive effect of C-peptide on α3(IV) collagen in podocytes (Figure 4). It was also confirmed that C-peptide dose-dependently induced activation of ERK in podocytes, as also reported previously in other cell-types [14,15,17,35]. These findings provide evidence that both G-protein coupling receptor and ERK play important roles in the effect of C-peptide in podocytes. Interestingly, growth-factor-activated ERK has been shown to inhibit signal transduction by TGF-β superfamily through phosphorylation of Smad1 and Smad2 in their linker domain [45–47]. Smad proteins phosphorylated by ERK lose their ability to translocate into the nucleus, and therefore are unable to initiate transcription upon ligand stimulation [48]. Whether ERK activation by C-peptide also results in phosphorylation of Smads in podocytes remains to be elucidated.

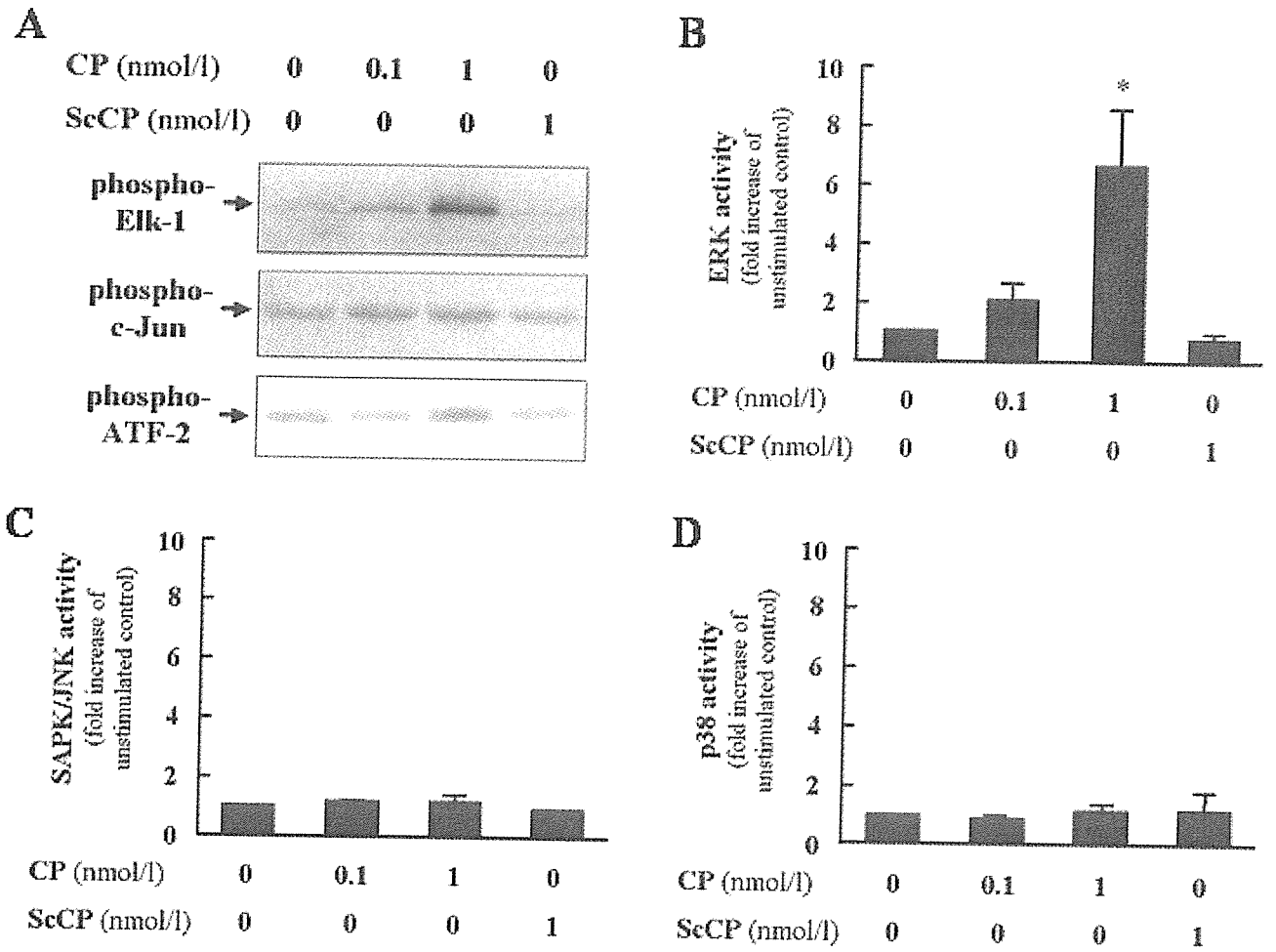

Figure 5. Effect of C-peptide on MAPK activity in podocytes. Podocytes were serum-starved for 16 h, treated with C-peptide (CP) or scrambled C-peptide (ScCP) for 5 min at the indicated doses, and lysed. ERK and p38 were then collected by immunoprecipitation, and SAPK/JNK was collected by adsorption to the c-Jun fusion protein and subjected to an *in vitro* kinase assay in the presence of the respective substrates, Elk-1 for ERK, ATF-2 for p38, and c-Jun for SAPK/JNK. Then the phosphorylation of the substrates was detected by western blotting with the specific antibodies against the phosphorylated substrates. Representative immunoblots are shown in (A). Results of densitometric scanning are presented as means ± SEM from three independent experiments (B, C, D). $^*P < 0.01$ *vs* the cells without C-peptide treatment

In some reports using myoblasts, adipocytes, or neurons, C-peptide was demonstrated to activate PI3K and NF-κB without activation of G-protein [49,50]. Therefore, there also is a possibility that C-peptide activates these molecules in podocytes. However, since PTX and PD98059 completely abolished the suppressive effect of C-peptide on TGF-β-induced type IV collagen expression, we concluded that at least this function depends on G-protein and ERK. Whether PI3K and NF-κB are involved in the renoprotective effect of C-peptide remains to be examined.

Our finding that C-peptide administration reduced urinary albumin excretion in STZ-diabetic mice is in keeping with previous observations in type 1 diabetic patients and STZ-diabetic rats [3–9]. The suppressive effect of C-peptide on albuminuria has usually been shown to associate with correction of glomerular hyperfiltration [3–7]. We, however, failed to observe significant correlation between creatinine clearance, a parameter closely related to glomerular filtration rate, and albumin excretion upon C-peptide administration. It maybe due to methodical limitation in measuring mouse creatinine clearance, which tends to give relatively large variance among individuals. At the same time, it is possible that reduction of albuminuria by C-peptide involves a mechanism independent of glomerular hemodynamics, e.g. through modification of matrix composition in GBM.

In conclusion, the present data provide a new perspective on the molecular effects of C-peptide on early diabetic glomerular changes by showing that C-peptide administration in diabetic mice reduces or prevents the urinary excretion of albumin and inhibits the up-regulation of type IV collagen in glomeruli. Moreover, the observed inhibitory effect of C-peptide on TGF-β-induced gene expression in cultured podocytes suggests a new therapeutic possibility to regulate the TGF-β signal.

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A patient with Werner syndrome and adiponectin gene mutation

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Abstract

Werner syndrome is a premature aging disease characterized by genomic instability and increased cancer risk. Here, we report a 45-year-old diabetic man as the first Werner syndrome patient found to have an adiponectin gene mutation. Showing graying and loss of hair, skin atrophy, and juvenile cataract, he was diagnosed with Werner syndrome type 4 by molecular analysis. His serum adiponectin concentration was low. In the globular domain of the adiponectin gene, I164T in exon 3 was detected. When we examined effects of pioglitazone (15 mg/day) on serum adiponectin multimer and monomer concentrations using selective assays, the patient's relative percentage increased in adiponectin concentration was almost same as that in the 18 diabetic patients without an adiponectin mutation, but the absolute adiponectin concentration was half of those seen in diabetic patients treated with the same pioglitazone dose who had no adiponectin mutation. The response suggested that pioglitazone treatment might help to prevent future Werner syndrome-related acceleration of atherosclerosis. Present and further clinical relevant to atherosclerosis in this patient should be imformative concerning the pathogenesis and treatment of atherosclerosis in the presence of hypoadiponectinemia and insulin resistance.

Keywords: Werner syndrome; Adiponectin mutation; Diabetes mellitus; Hypoadiponectinemia; Thiazolidine therapy

1. Introduction

Werner syndrome is an autosomal recessive hereditary disease characterized by premature aging, genomic instability, and accelerated atherosclerosis, and increased cancer risk [1,2]. The defective gene product in Werner syndrome belongs to the ReqQ family of DNA helicases [3]. Here, we report the first patient with Werner syndrome found to have an adiponectin gene mutation as well. We examined changes in adiponectin secretion in response to pioglitazone therapy.

2. Case presentation

A 45-year-old man was diagnosed with diabetes when cataract developed at the age of 25 years. He did not seek further treatment until he was 39 years old, when he was admitted to another hospital. There he was given insulin and was noted to have abdominal fat accumulation. He was referred to our hospital in April 2004.

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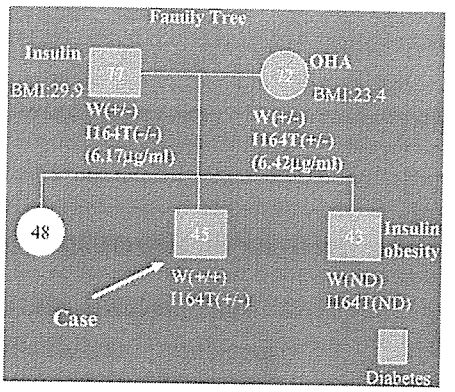

Fig. 1. The patient's father, mother, and uncle had diabetes; the father and uncle were treated with insulin, and the mother with oral hypoglycemic agents. Both parents were heterozygous for Werner syndrome type 4, and heterozygosity for the adiponectin gene mutation I164T was identified in the mother. Values shown are serum adiponectin concentrations (μg/ml).

The patient's father and uncle had diabetes; recently, his mother also had been diagnosed with diabetes. Fig. 1 shows the patient's family tree. His parents both were found to be heterozygous for the Werner mutation, while his mother was heterozygous for an I164T mutation in the adiponectin gene. No consanguinity was reported.

Height was 151.8 cm and weight was 38 kg. Blood pressure was 158/80 mmHg and the pulse was regular with a rate of 92 min^{-1}. The patient injected insulin before each meal (Penfil R 6U) and before sleep (Penfil N 6U). Hemoglobin (Hb) A1c was 6.7%; total serum cholesterol, high-density lipoprotein (HDL) cholesterol, and triglyceride concentrations were 208, 59, and 190 mg/dl, respectively. Urinary albumin excretion was 26.5 mg/g creatinine. The serum C-peptide concentration was 6.61 ng/ml with a simultaneous plasma glucose concentration of 156 mg/dl, suggesting that insulin secretory capacity was preserved and implying that insulin resistance was likely. The patient showed graying and loss of hair, skin atrophy, and juvenile cataract. We diagnosed him with Werner syndrome type 4 according to molecular analysis [4].

Yokote et al. [5], previously, reported serum adiponectin concentrations to be decreased in Werner syndrome (mean 3.1 μg/ml); our patient's serum adiponectin concentration was particularly low (2.24 μg/ml; to adiponectin monomer assay kit, Otsuka, Tokyo, Japan). When we sequenced the adiponectin gene, heterozygous mutation representing I164T in exon 3 was seen in the globular domain, as was demonstrated in his mother. This mutation has been reported to be atherogenic and to promote insulin resistance, leading to ischemic heart disease [6]. As the adiponectin and Werner genes are located on chromosome 3 and 8, respectively. We concluded that the two mutations were associated coincidentally.

To evaluate vascular atherosclerosis, carotid intima media thickness (IMT) was examined ultrasonographically. While this was only 0.6 mm, calcified plaques 2 mm in thickness were observed in right and left carotid arteries.

We next examined the effects of pioglitazone (15 mg/day) on adiponectin concentrations in the patient using separate adiponectin assay kits to detect the total monomers (Otsuka) and multimeric forms (Fujirebio, Tokyo, Japan). We compared his response to treatment with those in 18 diabetic patients whose adiponectin exon sequences were normal. Responses of serum adiponectin concentrations in the assay for monomers to 15 mg/day of pioglitazone in the other 18 diabetic patients were as follows: 5.68 ± 0.67 μg/ml before pioglitazone, 11.76 ± 1.85 μg/ml (at 1 month), and 11.81 ± 2.20 μg/ml (at 2 months, mean ± S.E.M.). In the Werner patient, the pretreatment adiponectin monomer concentration was 2.32 μg/ml; the1-month value, 6.07 μg/ml; the 2-month value, 5.02 μg/ml (Fig. 2A). Expressed relative to basal concentrations, responses of adiponectin monomer concentrations in

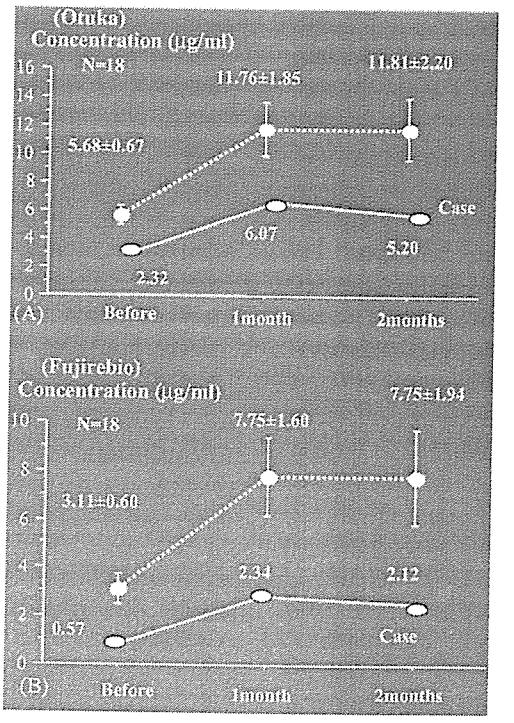

Fig. 2. A: Serum adiponectin concentrations (A, monomer; B, multimer) in response to 15 mg/day of pioglitazone. Data are shown for 18 diabetic patients without an adiponectin gene mutation (means ± S.E.M., broken line) and for the Werner patient (solid line).

the 18 patients with no mutation were 218.5 ± 16.1% (1 month) and 235.7 ± 16.2% (2 months). The Werner patient's relative responses were similar (261.6%, 1 month; 224.1%, 2 months). Serum adiponectin multimer concentrations in response to 15 mg/day of pioglitazone in the diabetic patients without an adiponectin mutation were as follows: 3.11 ± 0.60 μg/ml (pretreatment), 7.75 ± 1.60 μg/ml (1 month), and 7.75 ± 1.94 μg/ml (2 months); in the Werner patient, these, respectively, were 0.57, 2.34, and 2.12 μg/ml (Fig. 2B). For the multimeric form, relative responses in the18 patients were 284.8 ± 25.9% (1 month) and 326.4 ± 35.7% (2 months). In the Werner patient, these, respectively, were 410.5% and 371.9%.

3. Discussion

The adiponectin I164T mutation has been reported to interfere with adiponectin secretion in transfected cultured cells [7,8]. Kadowaki et al. reported that I164T adiponectin could not assemble into trimers, resulting in impaired secretion from the cell [7]. Another study using gel filtration reported that oligomerization was similar to that seen in wild-type adiponectin, but secretion from adipocytes into plasma was disrupted [8]. In our patient's response to pioglitazone, the serum adiponectin concentration was only half that seen in diabetic patients without mutation of the adiponectin gene, suggesting that secretion of mutant adiponectin from adipose tissues into plasma might be disturbed, and with only the wild-type adiponectin responding. The absolute change in serum concentration of adiponectin multimer, measured in response to pioglitazone, was slightly less than that of the monomer in the Werner patient compared with the other 18 diabetic patients, suggesting that processing of mutatnt adiponectin monomer to high-molecular-weight multimer might be compromised.

Here, we first reported a Werner syndrome patient with an additional mutation involving the adiponectin gene. Our study suggested that despite some differences between monomeric and multimeric forms, serum concentrations of both forms of adiponectin could be increased by treatment with thiazolidine derivatives in patients with hypoadiponectinemia resulting from a heterozygous adiponectin gene mutation. These and future data concerning long-term effects on atherosclerosis in this patient may be informative concerning the pathogenesis and treatment of atherosclerosis associated with hypoadiponectinemia and insulin resistance.

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