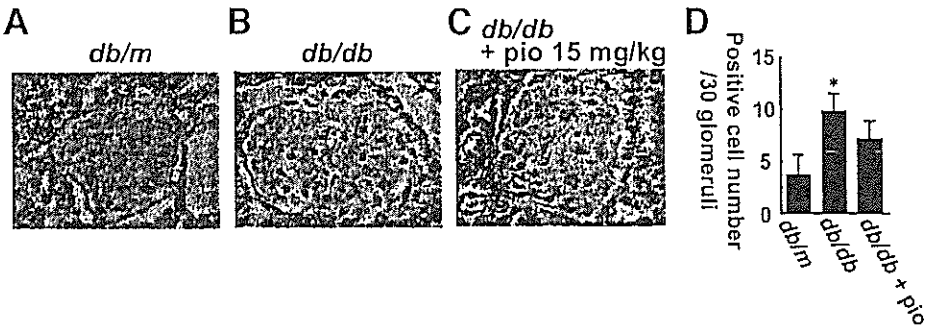

FIG. 4. Immunohistochemical analysis for HIF-1α expression in the glomeruli of *db/db* mice at 7 weeks of age. *A*: *db/m* mice. *B*: *db/db* mice. *C*: *db/db* mice plus 15 mg/kg pioglitazone (pio). *D*: HIF-1α–positive cell number in 30 glomeruli. Values are the means ± SE, $n = 4$ for each group. *$P < 0.05$ vs. *db/m*.

stage in this study (data not shown), ephrin B2 and HIF-1α mRNA were already upregulated at the normoalbuminuric stage and remained elevated at an early stage of diabetic nephropathy in isolated glomeruli of diabetic mice (Fig. 1). Because ephrin B2 and HIF-1α relate to angiogenesis, and because HIF-1α induces VEGF (40), elevation of ephrin B2 and HIF-1α may be an important early step for glomerular angiogenic change in diabetic nephropathy. GLEPP1 is related to podocyte differentiation (29), and its expression decreases in dedifferentiated podocytes (41). Pod-1 is one of the transcriptional factors important for glomerulogenesis and podocyte differentiation (24,28). Both ephrin B2 and HIF-1α are abundantly expressed in glomerular podocytes in the developing kidney (27,30). Taken together, podocyte injury may play a pivotal role in diabetic glomerulopathy, including glomerular angiogenic change. Other kidney development–related molecules (e.g., gremlin and transforming growth factor-β) were also suggested to contribute to the pathogenesis of diabetic nephropathy (42,43). Thus, the current study raises the possibility that the alteration of the kidney development–related molecules, particularly glomerulogenesis-related molecules (ephrin B2, HIF-1α, GLEPP1, and Pod-1), is a key mediator for diabetic glomerulopathy. This possibility is strengthened by our finding that high glucose induced a similar pattern of changes in glomerulogenesis-related gene expression in cultured murine podocytes because hyperglycemia is a well-known determinant of diabetic nephropathy.

Another new finding in the current study is that extracellular matrix and cell structure–related genes were differentially expressed at an early stage of diabetic nephropathy. This is consistent with the development of mesangial expansion several weeks later in this model. Among these genes, we focused on genes playing important roles in podocyte structure, i.e., actinin 4α and DG1. Actinin 4α is an actin–cross-linking protein, and mice with mutant actinin 4α revealed foot process fusion and podocyte vacuolization (31). DG1, a heavily glycosylated peripheral membrane protein located in podocytes, is thought to keep foot process shape, and it decreases in proteinuric renal diseases (32,44). Thus, the current study suggests that podocyte structure and function may already alter at an early stage of nephropathy. In contrast to previous reports, actinin 4α and DG1 mRNA expression increased in this study. Induction of these genes might reflect the glomerular repairing process, as reported in a puromycin aminonucleoside nephrosis model (45).

Insulin resistance is a major feature of type 2 diabetes, and it precedes the onset of microalbuminuria. Greater degrees of insulin resistance are evident when urinary albumin excretion is elevated in type 2 diabetes (15), and hyperinsulinemia in the pre-diabetic state may contribute to microalbuminuria in type 2 diabetes (1). In our microarray analysis, alteration in most of the development-related gene expression was restored by pioglitazone treatment with amelioration of albuminuria and hyperglycemia. Among them, the restoration of ephrin B2 and Pod-1 were confirmed by RT-PCR, and nuclear localization of HIF-1α was attenuated by pioglitazone. Although we could not evaluate the effect of pioglitazone on GLEPP1 gene expression because of its transient upregulation in this study, these results suggest that insulin resistance might be important in inducing the alteration in the expression of kidney development–related genes, including glomerulogenesis-related genes at early stages of nephropathy. Similarly, insulin resistance might also induce phenotype alteration of podocytes at an early stage of nephropathy because the upregulation of DG1 and actinin 4α genes was attenuated by pioglitazone treatment. We could not rule out the possibility that hyperglycemia per se directly altered glomerulogenesis-related gene expression because high glucose stimulated expression of glomerulogenesis-related genes in cultured podocytes and because administration of pioglitazone improved insulin resistance as well as hyperglycemia.

In conclusion, we demonstrated that the differential expression of glomerulogenesis-related genes already took

TABLE 4
Effects of pioglitazone treatment on body weight, blood glucose levels, and urinary albumin excretion

	Body weight (g)	Blood glucose levels (mg/dl)	Urinary albumin excretion (mg/16 h)	Urinary albumin excretion (μg/mg creatine)
Untreated *db/db*	37.1 ± 0.3	575 ± 25	32.5 ± 6.3	0.49 ± 0.13
db/db + 3 mg/kg pioglitazone	38.2 ± 0.2	521 ± 28	26.3 ± 4.0	0.42 ± 0.03
db/db + 15 mg/kg pioglitazone	41.7 ± 0.9*	295 ± 51*	12.6 ± 1.9*	0.21 ± 0.03*
db/m	25.4 ± 0.3	128 ± 12	5.2 ± 1.1	0.16 ± 0.02

Data are the means ± SE. Each group has $n = 12$. *$P < 0.01$ vs. untreated *db/db*.

place at the normoalbuminuric stage in the isolated glomeruli from *db/db* mice, whereas the expression of podocyte structure–related genes were altered at an early nephropathy stage with the elevation of microalbuminuria. We also showed that pioglitazone treatment restored most of the differential expression of glomerulogenesis- and podocyte structure–related genes. These findings suggest that the alteration of these genes might be relevant to the pathogenesis of diabetic glomerulopathy in type 2 diabetes with insulin resistance. Pioglitazone treatment even at the normoalbuminuric stage might be useful for the prevention of diabetic nephropathy.

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ARTICLE

Transgenic overexpression of brain natriuretic peptide prevents the progression of diabetic nephropathy in mice

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Abstract

Aims/hypothesis Brain natriuretic peptide (BNP) is a potent vasorelaxing and natriuretic peptide that is secreted from the heart and has cardioprotective properties. We have previously generated hypotensive transgenic mice (BNP-Tg mice) that overproduce BNP in the liver, which is released into the circulation. Using this animal model, we successfully demonstrated the amelioration of renal injury after renal ablation and in proliferative glomerulonephritis. Glomerular hyperfiltration is an early haemodynamic derangement, representing one of the key mechanisms of the pathogenesis of diabetic nephropathy. Based on the suggested involvement of increased endogenous natriuretic peptides, the aim of this study was to investigate their role in the development and progression of diabetic nephropathy.

Materials and methods We evaluated the progression of renal injury and fibrogenesis in BNP-Tg mice with diabetes induced by streptozotocin. We also investigated the effect of BNP on high glucose-induced signalling abnormalities in mesangial cells.

Results After induction of diabetes, control mice exhibited progressively increased urinary albumin excretion with impaired renal function, whereas these changes were significantly ameliorated in BNP-Tg mice. Notably, diabetic BNP-Tg mice revealed minimal mesangial fibrogenesis with virtually no glomerular hypertrophy. Glomerular upregulation of extracellular signal-regulated kinase, TGF-β and extracellular matrix proteins was also significantly inhibited in diabetic BNP-Tg mice. In cultured mesangial cells, activation of the above cascade under high glucose was abrogated by the addition of BNP.

Conclusions/interpretation Chronic excess of BNP prevents glomerular injury in the setting of diabetes, suggesting that renoprotective effects of natriuretic peptides may be therapeutically applicable in preventing the progression of diabetic nephropathy.

Keywords Diabetic nephropathy · Extracellular matrix · Glomerular hyperfiltration · Natriuretic peptide · Transforming growth factor-β · Transgenic mice

Abbreviations

ANP	atrial natriuretic peptide
BNP	brain natriuretic peptide
cGMP	guanosine 3′,5′-cyclic monophosphate
CNP	C-type natriuretic peptide
ERK	extracellular signal-regulated kinase
GC	guanylyl cyclase
MAPK	mitogen-activated protein kinase
PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate
Tg	transgenic

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Introduction

Among diabetic complications, an increasing prevalence of nephropathy is one of the most intractable and serious problems worldwide [1]. Diabetic nephropathy is the leading cause of end-stage renal disease in many countries,

and effective therapy to prevent progression at advanced stages remains unsatisfactory [1–3]. Hyperglycaemia is a necessary precondition for the development of diabetic renal lesions [4, 5], while systemic hypertension is an equally important aggravating factor of the disease [6]. Mechanisms including glomerular hypertension with hyperfiltration, renin–angiotensin system (RAS) activation, increased oxidative stress and advanced glycation end-products, activation of the protein kinase C (PKC) and mitogen-activated protein kinase (MAPK) pathways, growth factors and cytokines such as TGF-β, and genetic susceptibility have been identified as important deteriorating factors [2, 7, 8], but the precise mechanisms involved in the progression of diabetic renal injury remain elusive.

The natriuretic peptide family, consisting of atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and C-type natriuretic peptide (CNP) [9], possesses potent diuretic, natriuretic and vasorelaxing properties, thereby regulating blood pressure, body fluid homeostasis and cardiorenal function [10, 11]. ANP and BNP are secreted predominantly by the cardiac atrium and ventricle, respectively, in response to volume expansion and ventricular wall stress [9, 11]. Increased cardiac secretion of BNP, as well as ANP, has been demonstrated in patients with cardiovascular diseases such as congestive heart failure, hypertension and renal failure, serving as one of the compensatory mechanisms against disease progression [11, 12]. ANP and BNP share the same receptor, a particulate guanylyl cyclase (GC)-coupled receptor, or GC-A, and exert almost identical actions [13]. The peptides are thought to function, in general, to antagonise the RAS both systemically and locally [9].

Elevated plasma levels of ANP and BNP are reported in patients with diabetic nephropathy [14–16]. Plasma BNP and pro-BNP also serve as powerful risk markers for cardiovascular disease in patients with diabetic nephropathy [17]. Glomerular hyperfiltration, one of the key mechanisms of the pathogenesis of diabetic nephropathy [2], is an early haemodynamic derangement observed in diabetes, and the involvement of increased endogenous natriuretic peptides has been suggested. Indeed, in experimental diabetic nephropathy, the blockade of elevated plasma ANP attenuated glomerular hyperfiltration and urinary albumin excretion [18, 19]. In addition, acute infusion of ANP augmented urinary albumin excretion in diabetic patients with nephropathy [16]. These observations suggest that natriuretic peptides may play a causative role in glomerular hyperfiltration and diabetic glomerular injury.

Conversely, several reports have shown potential renoprotective effects of natriuretic peptides on various nephropathies. Administration of ANP exerted beneficial effects in experimental and clinical acute renal failure [20, 21]. We generated hypotensive transgenic mice overexpressing the mouse gene encoding BNP (*Nppb*) in the liver (BNP-Tg mice), which showed more than a 100-fold increase in plasma BNP and constitutive elevation of urinary guanosine 3′,5′-cyclic monophosphate (cGMP) levels [22]. Using this animal model, we successfully demonstrated amelioration of renal injury after renal ablation [23] and in proliferative glomerulonephritis [24]. However, the long-term effects of increased natriuretic peptides on diabetic renal injury remain unknown. The aim of the present study was to investigate the effects of a chronic excess of BNP on diabetic renal injury using streptozotocin-induced diabetes in BNP-Tg mice. We also studied the actions of BNP on cultured mesangial cells in the presence of high glucose.

Materials and methods

Animals All animal experiments were conducted in accordance with our institutional guidelines for animal research. Generation of BNP-Tg mice (line 55) harbouring 20 copies of the transgene under the control of the human serum amyloid P component promoter has been reported elsewhere [22–24]. This promoter is active only in the liver after birth [22]. BNP-Tg mice and their littermates, C57BL/6J non-transgenic mice (non-Tg mice), were 10 weeks of age at the beginning of this study. Mice were fed on standard chow (CE-2 containing 0.5% NaCl; Clea Japan, Tokyo, Japan) and given free access to water. We maintained the animals under alternating 12-h cycles of light and dark.

Induction of diabetes Diabetes was induced in mice by daily intraperitoneal injection of streptozotocin (70 mg/kg body weight, for 4-7 days) (Sigma, St Louis, MO, USA) in citrate buffer until the blood glucose level was raised to >16.7 mmol/l [25]. Control mice received citrate buffer only. Blood glucose was measured in tail vein blood using the *o*-toluidine method (Sigma kit) [25] under non-fasted conditions. Blood pressure was measured every 4 weeks by the indirect tail-cuff method [23]. Urine specimens (24 h) were obtained from each mouse every 4 weeks for measurement of creatinine and albumin [24]. Urinary albumin excretion was assayed with a murine albumin ELISA kit (Exocell, Philadelphia, PA, USA). Urinary and serum creatinine levels were measured using an enzymatic method (SRL, Tokyo, Japan) [24]. A subgroup of the diabetic non-Tg mice were administered hydralazine [23]; mice were given drinking water containing 60 mg/l hydralazine hydrochloride (Sigma) from 1 week after the induction of diabetes. Mice were killed after 16 weeks of diabetes under ether anaesthesia, and samples were collected for histological and biochemical analyses.

Renal histology and morphometric analysis Kidney sections were fixed by immersion in Carnoy's solution, followed by 4% buffered formaldehyde, and embedded in paraffin. Sections (2 μm thick) were stained with periodic acid–Schiff and examined by light microscopy. Measurement of the glomerular cross-sectional area and the mesangial area of 30 glomeruli randomly selected in each mouse by scanning of the outer cortex was performed with a computer-aided manipulator (KS-400; Carl Zeiss Vision, Munich, Germany) [23, 24].

Immunohistochemistry For immunohistochemical study of TGF-β, the kidney sections embedded in Optimal Cutting Temperature (OCT) compound (Sakura Finetechnical, Tokyo, Japan) were snap frozen in acetone/dry ice, and 4-μm-thick cryostat sections were fixed in acetone [23]. The sections were washed with phosphate-buffered saline, and treated with 0.9% H_2O_2 in methanol for 30 min to quench endogenous peroxidase activity. The specimens were incubated overnight at 4°C with rabbit anti-mouse TGF-β antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After incubation with biotin-conjugated second antibody, the specimens were processed by use of an avidin–biotin–peroxidase complex kit (Vector, Burlingame, CA, USA) and developed with 3,3′-diaminobenzidine tetrahydrochloride (Kanto Chemical, Tokyo, Japan).

Cell culture Mesangial cells were established from glomeruli isolated from 10-week-old male Sprague–Dawley rats using a differential sieving method [25, 26] and used at passages 7–10. Mesangial cells were identified by immunofluorescence techniques [26]. Cells were grown in DMEM (Gibco BRL, Grand Island, NY, USA) containing 20% FCS (Sanko Junyaku, Tokyo, Japan). As the cells reached 80% confluence, they were grown in DMEM containing 10% FCS supplemented with 5.6 mmol/l glucose (normal glucose), 25 mmol/l glucose (high glucose), or 5.6 mmol/l glucose plus 19.4 mmol/l mannitol (as an osmotic control) for 5 days. After this time, the medium was changed to DMEM containing 0.2% FCS supplemented with 5.6 mmol/l glucose, 25 mmol/l glucose, or 5.6 mmol/l glucose plus 19.4 mmol/l mannitol for 24 h. Then, in the presence or absence of rat BNP (100 nmol/l) (Peptide Institute, Osaka, Japan) or 8-bromo-cGMP (1 mmol/l) (Sigma), the cells were further incubated for 24 h. Phorbol 12-myristate 13-acetate (100 nmol/l) (PMA; Sigma) was used for the activation of PKC [26].

Northern blot analysis Total RNA was extracted from whole kidney and mesangial cells by the acid guanidinium–phenol–chloroform method and used for northern blot analysis as described previously [24, 25]. In brief, 25 μg of total RNA was electrophoresed on 1.1% agarose gels containing 2.2 mol/l formaldehyde, and RNA was transferred onto nylon membrane filters. The cDNA fragments corresponding to genes for rat TGF-β_1 (*Tgfb1*, nt 1142–1546), rat fibronectin (*Fn1*, nt 619–1082), mouse α1(IV) collagen (*Col4a1*, nt 5808–6165) and mouse TGF-β_1 (*Tgfb1*, nt 1141–1549), which were prepared by RT-PCR using rat and mouse kidney mRNA, were used as probes. The filter was hybridised with radiolabelled probes and autoradiography was performed using a BAS-2500 bioimaging analyser (Fuji Photo film, Tokyo, Japan). The filters were rehybridised with human *GAPDH* cDNA probe for normalisation.

Western blot analysis Whole-kidney tissues and mesangial cells were lysed on ice in lysis buffer containing 1 mol/l Tris–HCl (pH 7.5), 12 mmol/l β-glycerophosphate, 0.1 mol/l EGTA, 1 mmol/l pyrophosphate, 5 mmol/l NaF, 10 mg/ml aprotinin, 2 mmol/l dithiothreitol, 1 mmol/l sodium orthovanadate, 1 mmol/l phenylmethylsulfonyl fluoride, and 1% Triton X-100 [24]. The lysates were centrifuged at 15,000 ×*g* for 20 min at 4°C, and supernatants mixed with Laemmli's sample buffer (40 μg protein/lane) were separated by 12.5% SDS-PAGE

Table 1 Characteristics of control and diabetic mice at 16 weeks

	Control		Diabetes	
	non-Tg (*n*=5)	BNP-Tg (*n*=8)	non-Tg (*n*=10)	BNP-Tg (*n*=10)
Blood glucose (mmol/l)	8.5±1.7	9.8±1.0	27.2±1.8[b]	31.1±0.9[c]
Systolic blood pressure (mmHg)	112±3	90±2[b]	108±2	92±2[d]
Body weight (g)	31.9±1.2	34.4±1.5	27.4±1.0[b]	31.6±0.5[c]
Kidney weight (g)	0.20±0.01	0.21±0.01	0.22±0.01	0.20±0.01
Kidney/body weight (%)	6.30±0.46	5.93±0.30	7.96±0.47[a]	6.37±0.28[d]

Values are expressed as means±SEM
[a] $p<0.05$ vs control non-Tg mice
[b] $p<0.005$ vs control non-Tg mice
[c] $p<0.005$ vs control BNP-Tg mice
[d] $p<0.05$ vs. diabetic non-Tg mice

and electrophoretically transferred onto Immobilon filters. The filters were incubated with antibodies against total extracellular signal-regulated kinase (ERK)-1/2 or phosphorylated ERK-1/2 (New England Biolabs, Boston, MA, USA) for 2 h at room temperature, and immunoblots were developed with horseradish peroxidase-conjugated donkey anti-rabbit IgG (Bio-Rad, Richmond, CA, USA) and a chemiluminescence kit (ECL One Plus; Amersham, Arlington Heights, IL, USA).

Statistical analysis Data are expressed as means±SEM. Statistical analysis was performed by ANOVA followed by Scheffe's test. A p value of <0.05 was considered statistically significant.

Results

Characteristics of diabetic mice At baseline, there was no significant difference between BNP-Tg and non-Tg mice in terms of blood glucose levels (9.1±0.4 vs 8.5±1.1 mmol/l, n=10 per group), body weight or kidney weight (data not shown). After induction of diabetes, both BNP-Tg and non-Tg mice exhibited sustained hyperglycaemia, with no significant difference in blood glucose levels between them (Table 1). At baseline, the blood pressure of the BNP-Tg mice was 15–20 mmHg lower than that of the non-Tg mice, as observed previously [23, 24], and remained significantly lower during the study (Table 1). There were no significant blood pressure changes in the two groups following the induction of diabetes. At 16 weeks, diabetic non-Tg mice showed renal hypertrophy, as indicated by an increase in kidney weight per body weight, whereas BNP-Tg mice did not (Table 1).

Renal function and proteinuria of diabetic mice To evaluate the functional alterations in the kidneys of diabetic mice, we examined urinary albumin excretion and serum creatinine and urea nitrogen levels, together with calculated creatinine clearance. At baseline, there were no significant differences in these parameters between control non-Tg and control BNP-Tg mice (Fig. 1a; see [23]). After induction of diabetes, urinary albumin excretion of diabetic non-Tg mice markedly increased by 4 weeks, peaked at 12 weeks, and remained significantly elevated until 16 weeks (23.4± 6.4, 142.7±33.1, 264.3±46.5, 290.3±55.0, and 181.5± 22.7 μg/day at 0, 4, 8, 12 and 16 weeks, respectively, n=10). In diabetic BNP-Tg mice, on the other hand, the increase in urinary albumin excretion was much attenuated and significantly milder than that observed in diabetic non-Tg mice at 8, 12 and 16 weeks (31.1±6.6, 100.8±22.6, 136.6±24.5, 144.1±18.6, and 120.6±19.2 μg/day at 0, 4, 8, 12 and 16 weeks, respectively, n=10) (Fig. 1a). Urinary albumin excretion adjusted for creatinine was also significantly lower (~50%) in diabetic BNP-Tg mice than in diabetic non-Tg mice and was not significantly different from that seen in control non-Tg mice (Fig. 1b).

After 16 weeks of diabetes, non-Tg mice exhibited significantly increased serum creatinine and urea nitrogen levels, together with reduced creatinine clearance (Table 2). In contrast, these parameters were not significantly different from controls in diabetic BNP-Tg mice (Table 2). Thus, functional impairment became manifest at the chronic

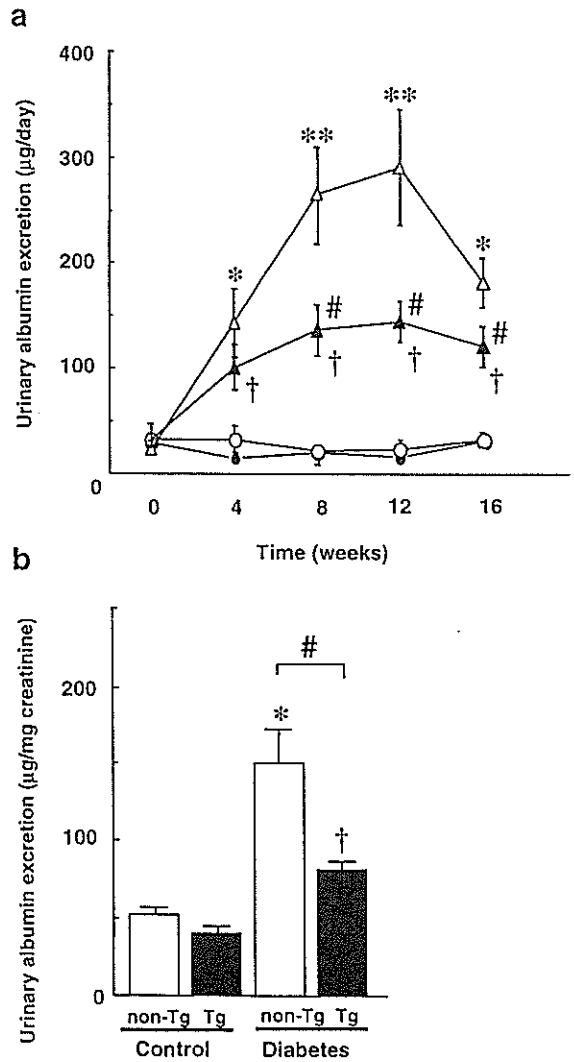

Fig. 1 a Daily urinary albumin excretion of control and diabetic mice at 0, 4, 8, 12 and 16 weeks after induction of diabetes. *Open circles*, control non-Tg mice (n=5); *closed circles*, control BNP-Tg mice (n=8); *open triangles*, diabetic non-Tg mice (n=10); *closed triangles*, diabetic BNP-Tg mice (n=10). b Urinary albumin excretion normalised to creatinine at 16 weeks. *p<0.05, **p<0.02 vs control non-Tg mice; †p<0.05 vs control BNP-Tg mice; #p< 0.05 vs diabetic non-Tg mice

Table 2 Renal function of control and diabetic mice at 16 weeks

Parameter	Control		Diabetes	
	non-Tg (n=5)	BNP-Tg (n=8)	non-Tg (n=10)	BNP-Tg (n=10)
Serum creatinine (μmol/l)	8.8±0.9	9.7±1.8	18.6±1.8[b]	12.4±0.9[c]
Blood urea nitrogen (mmol/l)	11.4±0.9	11.2±0.9	16.8±1.4[a]	14.6±1.6
Creatinine clearance (ml/min)	0.56±0.11	0.57±0.10	0.30±0.05[a]	0.54±0.06[c]

Values are expressed as means±SEM
[a]p< 0.05 vs control non-Tg mice
[b]p< 0.02 vs control non-Tg mice
[c]p< 0.05 vs diabetic non-Tg mice

phase in diabetic non-Tg mice, whereas renal function was well preserved in diabetic BNP-Tg mice.

Renal histology of diabetic mice We examined renal histological changes in diabetic non-Tg and BNP-Tg mice 16 weeks after induction of diabetes (Fig. 2). Compared with control (Fig. 2a), diabetic non-Tg mice showed

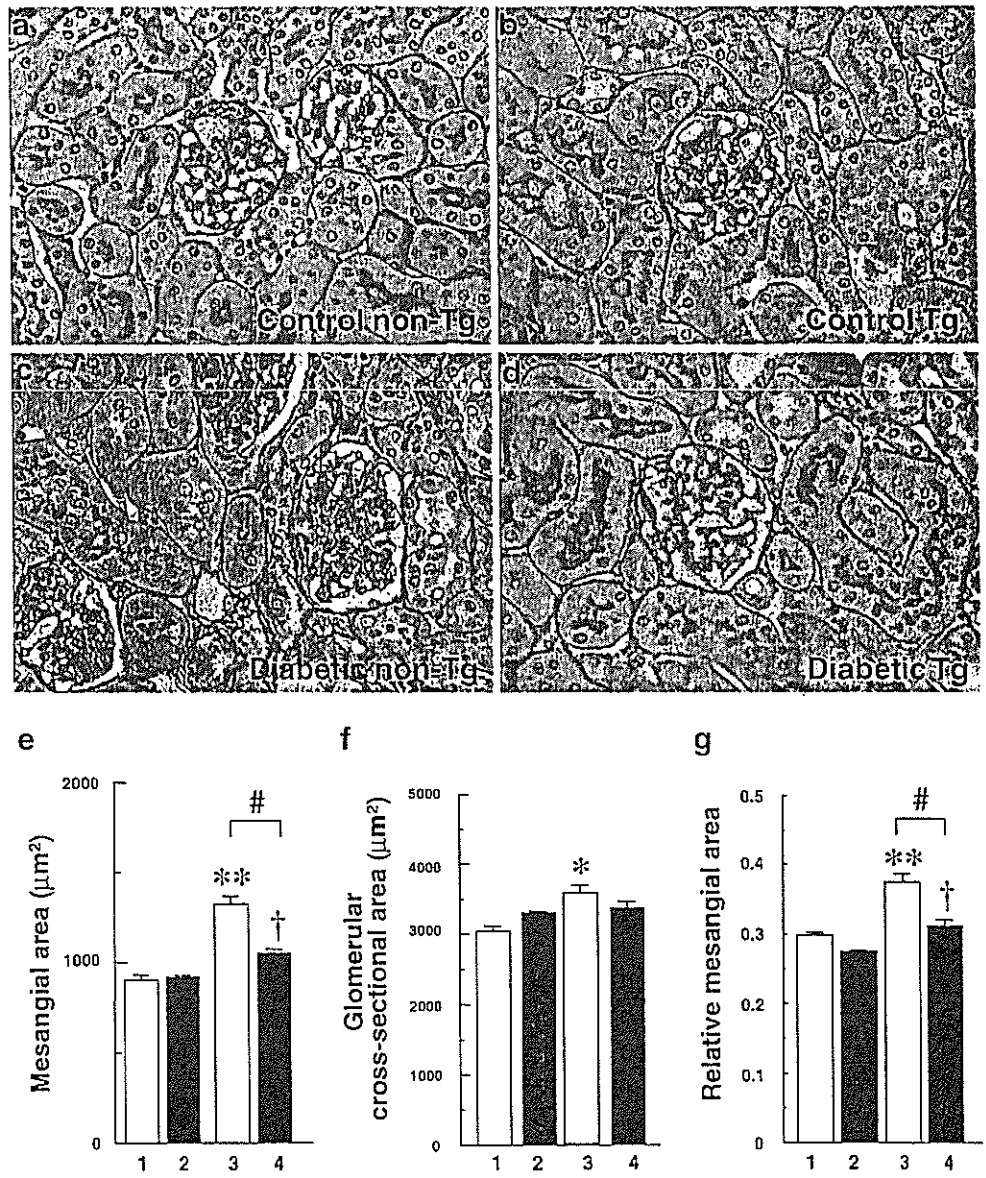

Fig. 2 Glomerular histology of diabetic mice at 16 weeks after induction of diabetes. Representative glomeruli from control non-Tg (a), control BNP-Tg (b), diabetic non-Tg (c), and diabetic BNP-Tg (d) mice are shown. Periodic acid–Schiff stain; magnification: ×400. Glomerular mesangial area (e), glomerular cross-sectional area (f) and relative mesangial area (g) in control and diabetic mice at 16 weeks. 1, control non-Tg mice (n=5); 2, control BNP-Tg mice (n=8); 3, diabetic non-Tg mice (n=10); 4, diabetic BNP-Tg mice (n=10) . Values are expressed as means±SEM. *p<0.05, **p<0.01 vs control non-Tg mice; †p<0.05 vs control BNP-Tg mice; #p<0.01 vs diabetic non-Tg mice

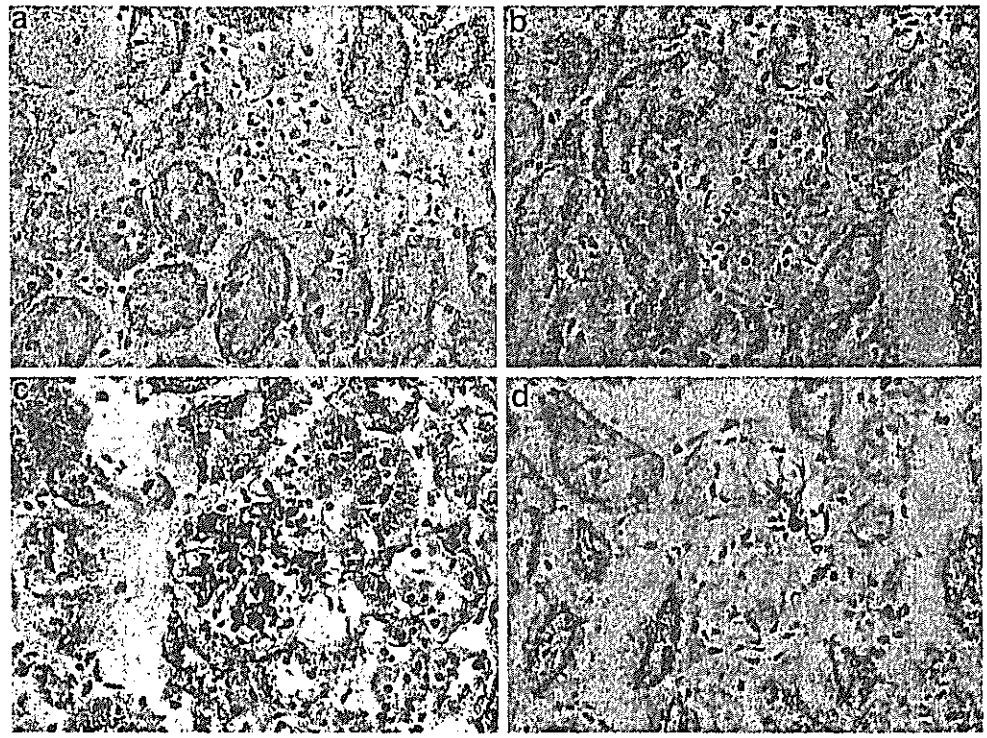

Fig. 3 Immunohistochemistry of TGF-β in control and diabetic mouse kidney at 16 weeks after induction of diabetes. Representative glomeruli from control non-Tg (a), control BNP-Tg (b), diabetic non-Tg (c), and diabetic BNP-Tg (d) mice are shown. Magnification: ×400

marked mesangial expansion and glomerular hypertrophy, along with partial tubular atrophy (Fig. 2c). In contrast, renal histology of diabetic BNP-Tg mice revealed only minimal glomerular hypertrophy and mesangial expansion with almost intact tubules (Fig. 2d), and was virtually indistinguishable from that of control BNP-Tg mice (Fig. 2b). Quantitative analysis revealed that the increase in mesangial area was marked in diabetic non-Tg mice, whereas this increase was significantly suppressed in diabetic BNP-Tg mice (1,328±58 vs. 1,037±21 μm^2, $p<0.01$, $n=10$ per group) (Fig. 2e). Glomerular hypertrophy was evident in diabetic non-Tg mice compared with control non-Tg mice (3,574±107 vs 3,048±66 μm^2, $p<0.05$), but was not apparent in BNP-Tg mice (3,378±103 vs 3,313± 30 μm^2 for diabetic vs control, respectively) (Fig. 2f). Accordingly, the increase in the relative mesangial area (mesangial area per glomerulus) was significantly blunted in diabetic BNP-Tg mice (Fig. 2g). Among the control mice, the mean glomerular area was slightly larger in the BNP-Tg mice than the non-Tg mice, but this difference was not statistically significant (Fig. 2f). These results indicate that the renal histological changes characteristic of diabetic nephropathy were prevented to a great extent in BNP-Tg mice.

Renal TGF-β1 expression The upregulation of TGF-β is postulated to play a pivotal role in facilitating extracellular matrix accumulation and subsequent glomerulosclerosis in diabetic glomerular injury [27, 28]. We therefore examined renal expression of the genes for TGF-β_1 (*Tgfb1*) and α1 (IV) collagen (*Col4a1*) in diabetic mice. By immunohistochemistry, we found that levels of TGF-β were markedly elevated in diabetic non-Tg mice (Fig. 3c) compared with control non-Tg mice (Fig. 3a), especially in the mesangial area. In diabetic BNP-Tg mice, on the other hand, this increase was significantly attenuated (Fig. 3d vs Fig. 3b). Likewise, northern blot analyses for *Tgfb1* and *Col4a1* revealed 3.5- to 4-fold increases in diabetic non-Tg mice compared with control ($p<0.05$, $n=7$) (Fig. 4a,b), whereas this upregulation was significantly reduced in diabetic BNP-Tg mice (1.3-fold increase relative to control BNP-Tg mice, $p<0.05$ vs diabetic non-Tg mice) (Fig. 4b). Thus, glomerular activation of the TGF-β-extracellular matrix protein cascade was significantly inhibited in diabetic BNP-Tg mice.

Renal ERK activation Accumulating evidence indicates that activation of the ERK/MAPK signalling pathway plays a key role in the induction of *Tgfb1* and extracellular matrix accumulation in diabetic nephropathy [29–31]. To address the mechanisms by which *Tgfb1* and matrix gene expression was inhibited in BNP-Tg mice, we investigated the phosphorylation of ERK in the kidney. Although ERK phosphorylation was minimal in the kidney tissues of both non-Tg and BNP-Tg mice under basal conditions, we found that levels of phosphorylated ERK were significantly increased in kidneys of diabetic mice (Fig. 5a). Importantly, phosphorylation of ERK in vivo was significantly lower in kidneys of diabetic BNP-Tg mice relative to diabetic non-Tg mice (Fig. 5b).

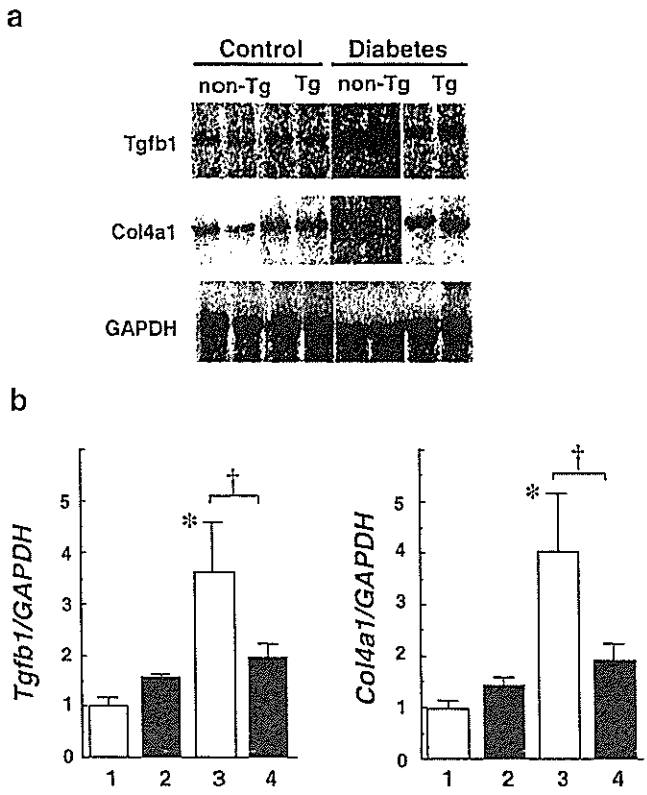

Fig. 4 Representative northern blots for *Tgfb1* and *Col4a1* mRNA expression (a) and their quantitative analysis (b) in kidney of control and diabetic mice at 16 weeks after induction of diabetes. 1, control non-Tg mice (n=4); 2, control BNP-Tg mice (n=4); 3, diabetic non-Tg mice (n=7); 4, diabetic BNP-Tg mice (n=7). Values are expressed as means±SEM. *p<0.05 vs control non-Tg mice. †p<0.05

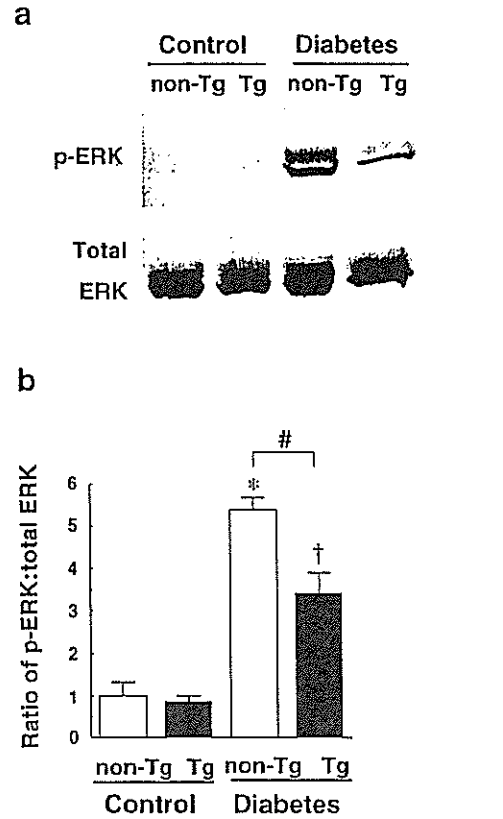

Fig. 5 ERK phosphorylation in the kidney of diabetic mice. Representative western blots of phosphorylated ERK-1/2 and total ERK-1/2 at 16 weeks after induction of diabetes (a) and quantitative analysis of phospho-ERK:total ERK (b). Values are expressed as means±SEM for n=4 in each group. *p<0.01 vs control non-Tg mice; †p<0.01 vs control BNP-Tg mice. #p<0.01

Effects of hydralazine administration Analyses thus far have suggested that a chronic excess of BNP prevents the progression of diabetic renal injury. Systemic blood pressure reduction is crucial to retard the progression of renal and vascular complications in diabetes [6, 32]. In order to explore whether the beneficial effects observed in BNP-Tg mice were the result of systemic hypotension, we studied the effect of hydralazine administration in diabetic non-Tg mice. Despite an effective reduction in systemic blood pressure to a level comparable to that in diabetic BNP-Tg mice (Fig. 6a), this treatment failed to prevent the increase in albuminuria (Fig. 6b) or to alleviate renal histological changes (data not shown). These results indicate that systemic hypotension in BNP-Tg mice does not play an important role in the renoprotective effects observed.

Effects of BNP in cultured mesangial cells under high glucose We next addressed the direct actions exerted by BNP using cultured mesangial cells under high glucose conditions. First, we investigated the effects of BNP on the expression of *Tgfb1* and the gene encoding its downstream effector, fibronectin. High glucose conditions significantly augmented *Tgfb1* and *Fn1* mRNA expression (1.8-fold and 1.5-fold of control [normal glucose, no BNP], respectively) (Fig. 7a,b). This effect was not simply due to high osmotic conditions, because an osmotic control with mannitol had no effect on *Tgfb1* mRNA expression (1.7-fold of control with high glucose vs 1.1-fold with mannitol, p<0.05) (Fig. 7c). This upregulation of *Tgfb1* and *Fn1* under high glucose conditions was effectively abolished by the addition of BNP (1.2-fold and 1.0-fold of control, respectively, p<0.01), or with a membrane-permeable analogue of cGMP (1.1-fold and 0.9-fold of control, respectively, p<0.01) (Fig. 7a,b).

We further examined the effects of BNP and cGMP on ERK phosphorylation of mesangial cells under high glucose conditions. High glucose-induced ERK phosphorylation in mesangial cells was significantly inhibited by the addition of BNP or cGMP (Fig. 8a). Moreover, BNP and cGMP effectively prevented ERK phosphorylation induced by PKC activation with PMA (Fig. 8b). Taken together, these results suggest that BNP inhibited PKC-ERK pathway activation and subsequent matrix gene activation, at least in part, locally in the mesangium in vivo, thereby leading to the amelioration of diabetic renal injury.

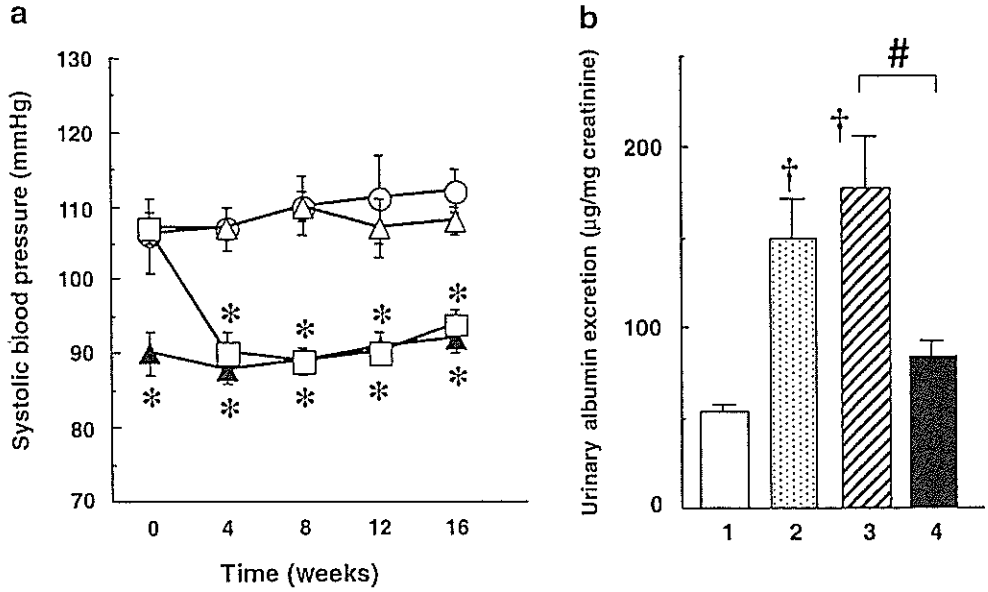

Fig. 6 a Effects of hydralazine administration on systolic blood pressure. *Open circles*, control non-Tg mice (n=5); *open triangles*, diabetic non-Tg mice (n=10); *closed triangles*, diabetic BNP-Tg mice (n=10); *open squares*, hydralazine-treated diabetic non-Tg mice (n=7). *p<0.005 vs diabetic non-Tg mice. b Urinary albumin excretion normalised to creatinine at 16 weeks. 1, control non-Tg mice; 2, diabetic non-Tg mice; 3, hydralazine-treated diabetic non-Tg mice; 4, diabetic BNP-Tg mice. †p<0.02 vs control non-Tg mice; #p<0.05

Discussion

Previous reports have suggested that increased natriuretic peptides may affect the course of diabetic nephropathy. Acute inhibition of elevated plasma ANP decreased hyperfiltration and albuminuria in experimental models [18, 19]. In patients with early-stage type 1 diabetes, ANP concentration was correlated with glomerular filtration rate [33]. Furthermore, acute ANP infusion increased the glomerular filtration rate, filtration fraction and albuminuria [34]. These studies investigated early haemodynamic abnormalities or only short-term effects of ANP (within several hours). Thus, the aim of this study was to assess the long-term effects of natriuretic peptides on glomerular function and histology. In order to address these issues, we investigated the effects of a chronic excess of BNP on the progression of diabetic nephropathy using BNP-Tg mice.

In this study, we demonstrate that transgenic overexpression of BNP prevents the progression of diabetic nephropathy in mice. In diabetic BNP-Tg mice, the accumulation of mesangial matrix was only minimal, with virtually no glomerular hypertrophy, in contrast to the histology of diabetic non-Tg mice (Fig. 2). Consistent with such histological amelioration, albuminuria was significantly attenuated in BNP-Tg mice, and renal function was well preserved (Fig. 1, Table 2). Although absolute creatinine values may be less accurate than those measured by HPLC, assessment of renal function in these groups should be valid considering the changes in serum creatinine essentially paralleled those in urea nitrogen (Table 2). These findings provide the first evidence that a chronic excess of BNP prevents the kidney from developing diabetic renal injury. We also demonstrated that the upregulation of *Tgfb* expression and protein levels in diabetic kidneys were markedly inhibited in BNP-Tg mice (Figs 3, 4). Considering a pathogenic role for TGF-β in cellular dysfunction, fibrogenesis and glomerular hypertrophy in diabetes [3, 28, 35], it is conceivable that the inhibition of renal TGF-β system activation contributed significantly to the observed protective effects of BNP from diabetic renal injury. Although the effect of BNP on glomerular haemodynamics was not investigated, it may be possible that the chronic excess of BNP lessened glomerular hypertension by inhibiting the RAS and TGF-β system tonically. It has been shown that combined inhibition of both angiotensin-converting enzyme and neutral endopeptidase, which potentiates ANP and BNP, results in lower glomerular capillary pressure than the former alone in subtotally nephrectomised rats [36].

There is no doubt that tight blood pressure control is important for retarding or preventing the progression of diabetic renal injury [6, 32]. Clearly, the diabetic BNP-Tg mice showed lower blood pressure. Although systemic blood pressure reduction with hydralazine treatment failed to alleviate nephropathy in diabetic non-Tg mice (Fig. 6), precise blood pressure profiles should have been different between the two hypotensive groups. We therefore cannot exclude the possibility that low blood pressure could account for the observed renoprotective effect in BNP-Tg mice.

ERK plays a pivotal role in activating mesangial TGF-β expression/signalling and extracellular matrix accumulation [29, 30]. We clearly show that ERK activation in the kidney tissue was significantly attenuated in BNP-Tg mice (Fig. 5).

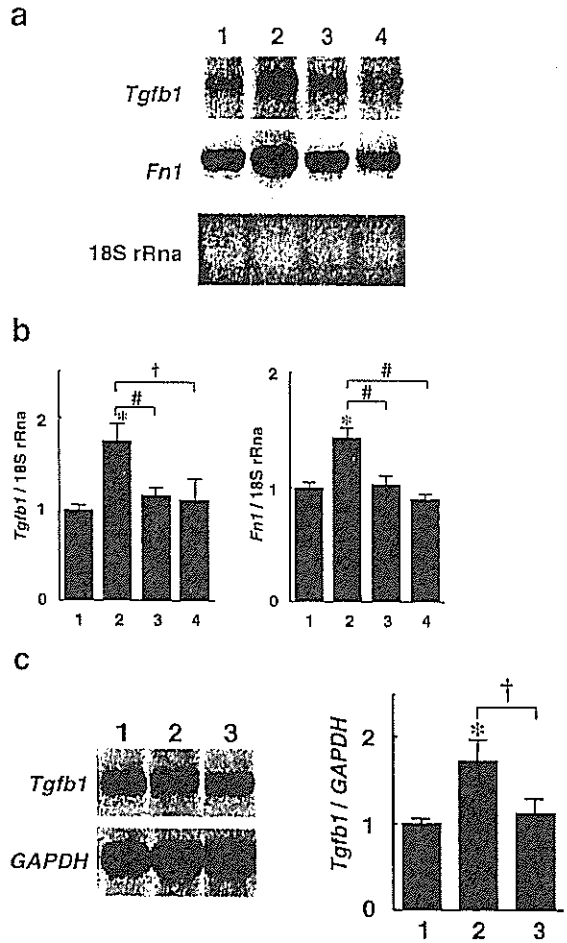

Fig. 7 Representative northern blots for *Tgfb1* and *Fn1* mRNA expression (a) and their quantitative analysis (b) in cultured mesangial cells under high glucose conditions. 1, normal glucose (5.6 mmol/l); 2, high glucose (25 mmol/l); 3, high glucose+BNP 100 nmol/l; 4, high glucose+cGMP 1 mmol/l. Values are expressed as means±SEM for n=6 in each group. $^{*}p<0.01$ vs normal glucose; $^{\dagger}p<0.02$; $^{\#}p<0.01$. c. Representative northern blots for *Tgfb1* mRNA expression and quantitative analysis in cultured mesangial cells. 1, normal glucose (5.6 mmol/l); 2, high glucose (25 mmol/l); 3, normal glucose+mannitol (19.4 mmol/l). Values are expressed as means±SEM for n=4 for each group. $^{*}p<0.02$ vs normal glucose. $^{\dagger}p<0.05$

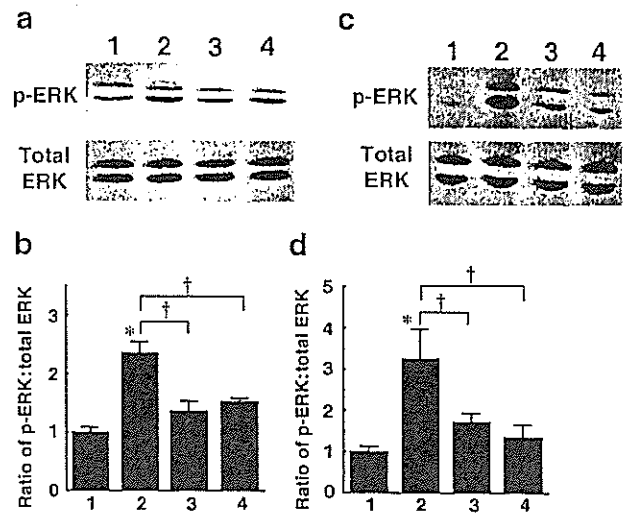

Fig. 8 a, b Inhibitory effects of BNP on ERK phosphorylation in cultured mesangial cells under high glucose conditions. 1, normal glucose (5.6 mmol/l); 2, high glucose (25 mmol/l); 3, high glucose+BNP 100 nmol/l; 4, high glucose+cGMP 1 mmol/l. Values are expressed as means±SEM for n=4 in each group. $^{*}p<0.01$ vs normal glucose; $^{\dagger}p<0.01$. c, d. Inhibitory effects of BNP on PMA-induced ERK phosphorylation. 1, control; 2, PMA 100 nmol/l; 3, PMA 100 nmol/l+BNP 100 nmol/l; 4, PMA 100 nmol/l+cGMP 0.1 mmol/l. Values are expressed as means±SEM for n=4 in each group. $^{*}p<0.01$ vs control; $^{\dagger}p<0.01$

We also demonstrated that BNP effectively inhibited ERK phosphorylation, as well as TGF-β expression, in cultured mesangial cells under high glucose conditions (Figs 7, 8). High glucose conditions activate PKC, and ERK activation occurs through a PKC-dependent mechanism [31, 37]. BNP also attenuated PKC-induced phosphorylation of ERK. It has been reported that ANP inhibits MAPK activation downstream of PKC via MAPK phosphatase activation in a cGMP-dependent manner [37]. These results therefore suggest that BNP exerts renoprotective effects in diabetes at least partly by locally inhibiting activation of the PKC-ERK pathway at the mesangium. The pathogenic role of PKC in matrix gene activation, haemodynamic abnormalities and proteinuria in vivo has already been shown in diabetic rodent models using a PKC β inhibitor [38, 39].

Podocytes are highlighted as the key in maintaining normal glomerular function and structure [40, 41], and podocyte loss or injury is closely associated with diabetic glomerular injury [42]. Natriuretic peptides act on podocytes and may modulate their function [43], but how natriuretic peptides affect podocytes in diabetic states or whether BNP exerted beneficial effects against podocyte injury in the current study are unclear. Further studies are needed to explore the effects of BNP on podocytes during the course of diabetic nephropathy.

The effect of BNP on glycaemic control is another issue to be addressed. Natriuretic peptides generally act to antagonise the systemic and local actions of angiotensin II [9]; they are therefore considered as endogenous RAS inhibitors. Growing evidence suggests that inhibition of the RAS exerts a beneficial effect on glycaemic control in experimental models and in clinical studies [44–46]. In the present study, we found no significant difference in blood glucose levels between non-Tg and BNP-Tg mice in this type of insulin-deficient model (Table 1). Whether natriuretic peptides or agonists of this system are beneficial in terms of regulating glycaemic control and preventing diabetic complications requires further investigation.

We have previously reported that BNP-Tg mice with higher copy numbers of the transgene show marked skeletal overgrowth [47], indicating that BNP likely activates the

physiological CNP/GC-B pathway in the bone to stimulate endochondral ossification [48]. Therefore, it is important to clarify whether the beneficial effects of BNP observed in this study are GC-A-dependent or GC-B-dependent. In the kidney, GC-A is localised in the mesangium, capillary and tubules, whereas GC-B is localised in the tubular system [11]. In the present study, BNP inhibited glomerular ERK activation and *Tgfb1* expression, suggesting that the effects of BNP were mediated via GC-A. However, it should be noted that the cultured mesangial cells used in our study express both GC-A and GC-B, where CNP has potent antifibrotic effects [24]; therefore the observed in vitro effects of BNP might also be exerted via GC-B. Analyses of crosses between BNP-Tg mice and GC-A null mice [10] and other combinations would answer these questions.

In summary, we demonstrate that a chronic excess of BNP in mice prevents diabetic glomerular injury, with amelioration of albuminuria and renal dysfunction, and these effects may be beyond those from mere systemic blood pressure reduction. Although we need to be cautious in interpreting these results and extrapolating them to clinical situations, our study opens up the possibility that the renoprotective effects of natriuretic peptides may be therapeutically applicable for the prevention of progression of diabetic nephropathy.

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Q7 日本人に適した運動は？

▶A 日本人に特に適している運動があるわけではないが，歩くことを主として比較的軽い全身運動を，なるべく毎日 30 分程度行うことが勧められる。

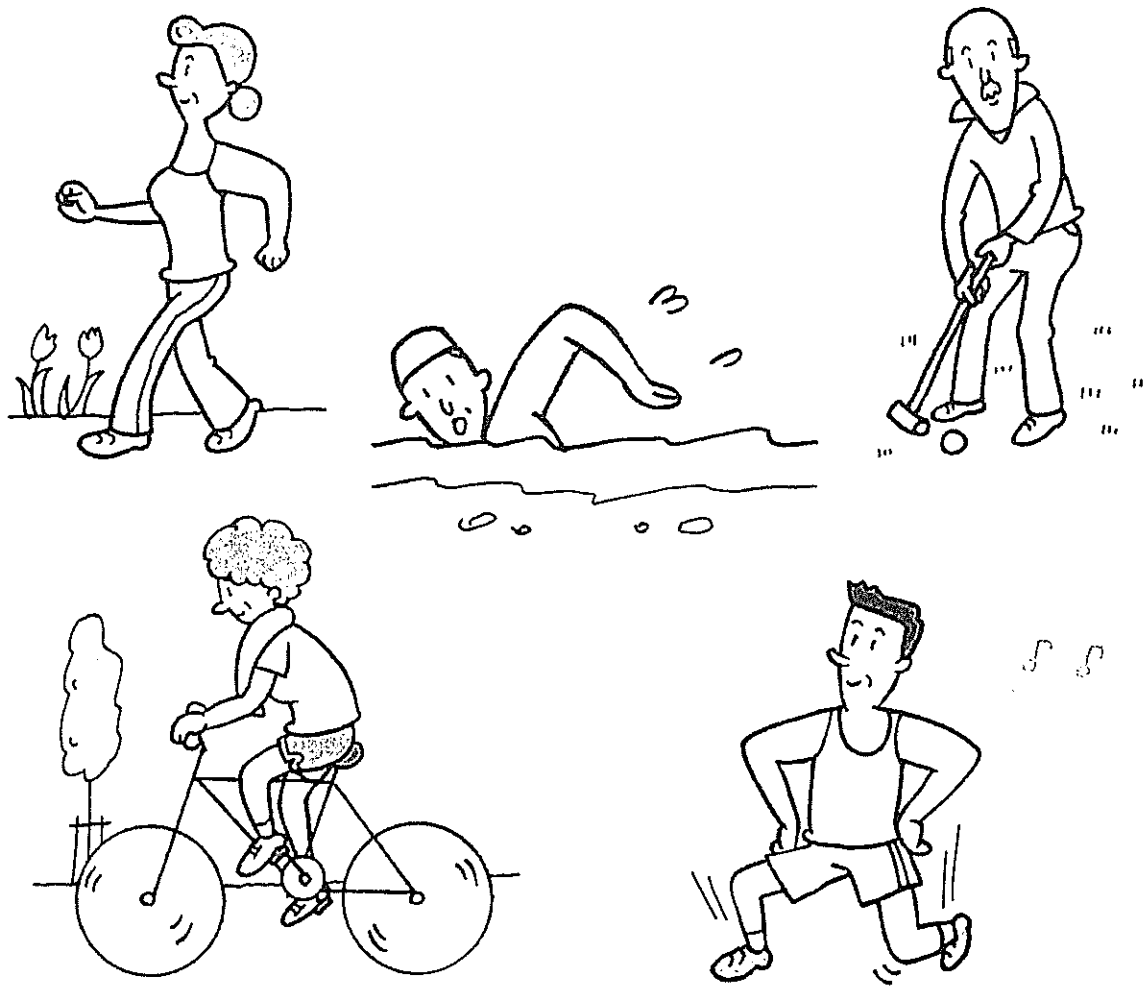


図１　高血圧者に勧められる運動の例[1)]

ウォーキングやサイクリングのような比較的軽い全身運動（有酸素運動）をなるべく毎日行うことが望ましい。

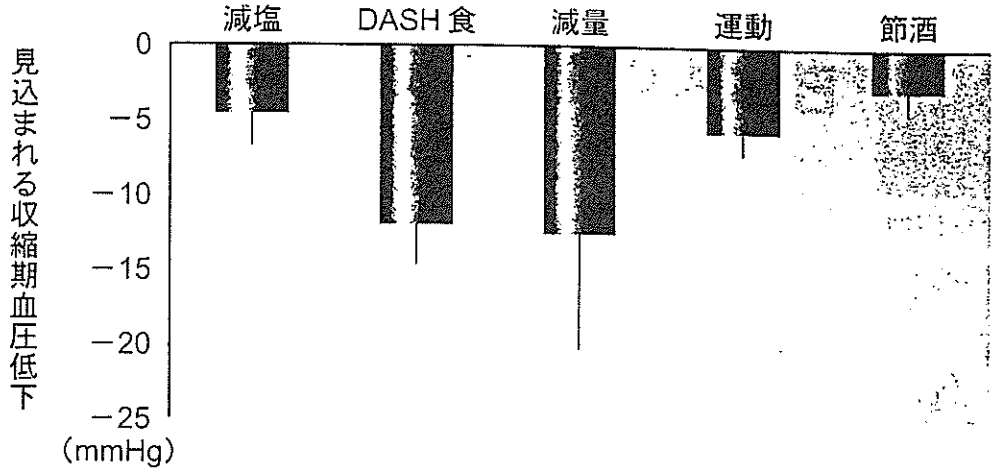


図2　生活習慣修正に基づく降圧の程度[2)]

減塩は食塩摂取量6 g/日の減少で概算，DASH食は野菜，果物，低脂肪乳製品に富む食事，減量は体重10 kgの減少で概算，運動は30分の早歩きを毎日施行，節酒は男性30 mL/日以下，女性15 mL/日以下のアルコール制限である。

運動は血圧を下げる効果があり，高血圧の治療についてのおもなガイドラインも非薬物療法の一つとしてこれを勧めている。高血圧者には，ウォーキングやサイクリングのような比較的軽い有酸素運動を定期的に（30分以上をなるべく毎日）行うことが勧められる[1~3)]（図1）。しかし，日本人に特に適した運動があるわけではない。

運動は減量にも有効であるが，これとは独立して血圧を下げる効果が認められている。運動をきちんと行えば血圧は5～10 mmHg低下し，糖・脂質代謝も改善する（図2）。また，よく歩く人や運動習慣がある者は，将来の循環器病や死亡率が少ないことが示されており，この効果は，年齢や肥満の有無に関わらず認められている。最近の報告では，身体活動量が多い老年者は認知症になりにくいことも明らかにされている。

ただし，運動中は血圧が上昇し，激しい運動では50 mmHg以上にもなる。高血圧治療における運動については，ある程度血圧がコントロールされてから始めることと，運動負荷検査を行うことが望ましい。体を動かさずに力を込めるウェイトトレーニングのようなものは，血管抵抗の増加とともに収縮期およ

び拡張期血圧が著しく上昇するのであまり好ましくない。全身的な有酸素運動のほうが好ましく，ウォーキングやスイミング，サイクリング，エアロビクス体操などがあげられる。これらは心拍出量と収縮期血圧は上昇するが，血管抵抗と拡張期血圧はあまり変わらない。

高血圧の人には，激しい運動を短時間するよりも，比較的軽い運動を長めに行うことが勧められる。適当な運動の強度は個人の体力や年齢などによって異なるが，一般的には少し動悸を感じる程度，もしくは心拍数が毎分100から120程度が目安になるであろう。運動はほとんどの高血圧者に推奨できるが，各個人の心血管疾患や骨関節疾患などに注意を払う必要がある。また，季節や時刻にも留意を要する。冬の寒い朝にいきなり走り出したり，夏の暑い日に水分をあまりとらずに運動を続けることは，心血管事故を誘発する可能性がある。

（河野雄平）

白衣高血圧の診断と治療

河野雄平　国立循環器病センター高血圧腎臓内科

白衣高血圧
随時血圧
白衣効果
白衣現象
家庭血圧
自由行動下血圧モニタリング

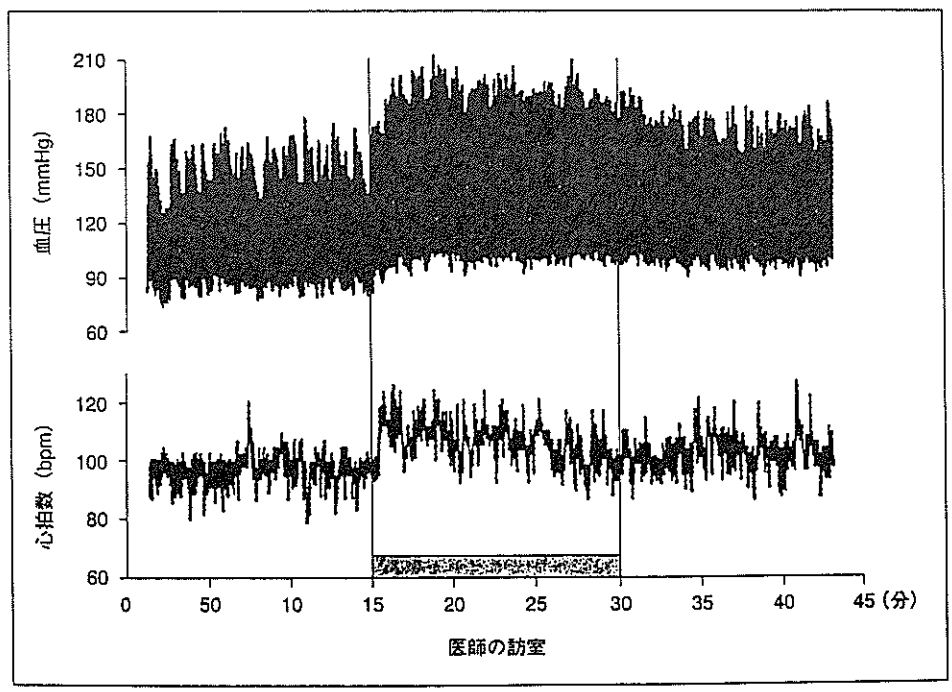
通常の血圧は正常であるが診察室，特に医師の前では高血圧を呈する白衣高血圧は以前よりよく知られており，随時血圧で高血圧と診断される者のかなりの部分を占めている。また，白衣効果や白衣現象とよばれる受診時の血圧上昇は，真の高血圧者においても高頻度に認められる。これらの診断には，家庭血圧測定あるいは自由行動下血圧モニタリング(ambulatory blood pressure monitoring：ABPM)が不可欠である。白衣高血圧が有害か否か，治療を要するかどうかについては議論が続いているが，ここでは白衣高血圧の病態やその診断と管理について概説する。

白衣高血圧と白衣効果

白衣高血圧とは，通常は正常血圧であるが診察室などの医療環境，特に医師の前では高血圧を呈する状態である。その頻度は診断基準などにより異なるが，随時血圧で高血圧と判定される者の約20%と考えられる[1]。

また，白衣効果あるいは白衣現象とよばれる診察室での血圧上昇は，白衣高血圧者に限らず真の高血圧者にも高頻度に認められる[2,3](図1)。筆者らの検討では，未

図1 ▶ 医師の訪室による血圧上昇と心拍数増加(白衣効果)



(文献3より引用)

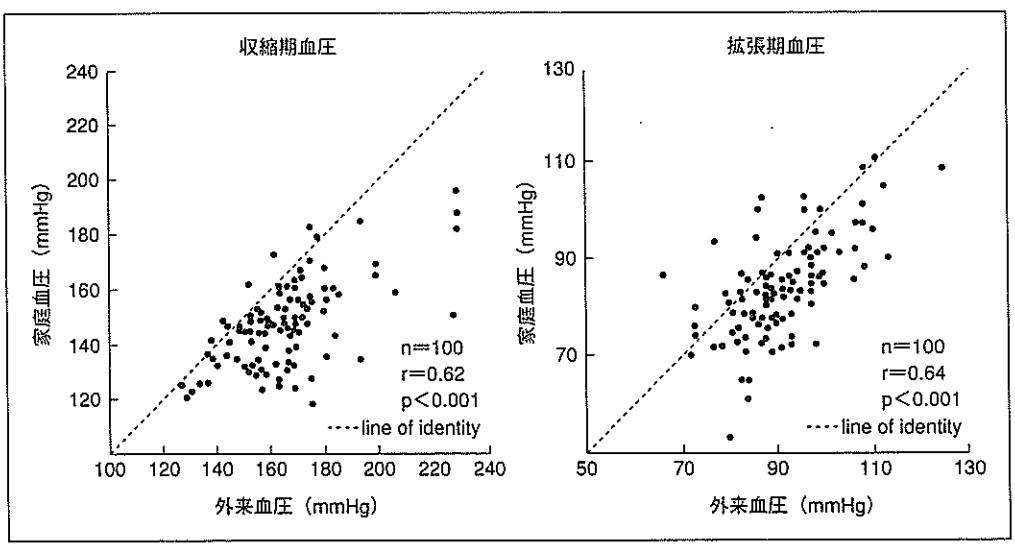
治療の患者の大部分は外来血圧が家庭血圧より高値で，その差は個人差が大きく，平均10/5mmHg程度であった[4]（図2）。また，治療中の高血圧患者の約20%は，外来血圧が家庭血圧より20/10mmHg以上高値であった[5]（表1）。ただし，白衣効果は随時血圧と自由行動下血圧あるいは家庭血圧との差として評価されることが多いが，その場合には受診時の血圧上昇のほかに日内変動や生活習慣，薬剤などの影響を受けることに注意を要する[2,3]。

白衣高血圧，白衣効果の機序

白衣高血圧や白衣効果の機序については，解明されているわけではないが，医療従事者特に医師への，また血圧測定への，条件づけられた警鐘反応が重要と考えられている[1,6]。すなわち，初めての血圧測定時には血圧は多少上昇する。血圧測定が繰り返されると，多くは慣れにより血圧上昇は小さくなる。しかし，一部の者は条件づけにより血圧上昇が同様にあるいは増幅されて持続し，白衣高血圧や白衣現象を呈すると考えられる（図3）。血圧測定や医師の存在が悪い知らせとして認識され，患者の血圧を繰り返して上昇させるのであろう。

白衣高血圧者における血圧上昇は医療環境下に限られることが多く，日常生活における血圧変動はそれほど大きくはない[6,7]（図4）。したがって，種々の刺激に対する昇圧反応が過剰であるとの考えは否定的と思われる。

図2 ▶ 未治療の患者の外来血圧と家庭血圧



（文献4より引用）

表1 ▶ 治療中の高血圧患者における明らかな白衣効果および逆白衣効果の頻度

外来－家庭血圧	All subjects (n=315)	<60歳 (n=105)	≧60歳 (n=210)
≧20/10mmHg	56 (18%)	22 (21%)	34 (16%)
≧40/20mmHg	3 (1%)	1 (1%)	2 (1%)
≦−20/−10mmHg	11 (3%)	4 (4%)	7 (3%)

（文献5より引用）

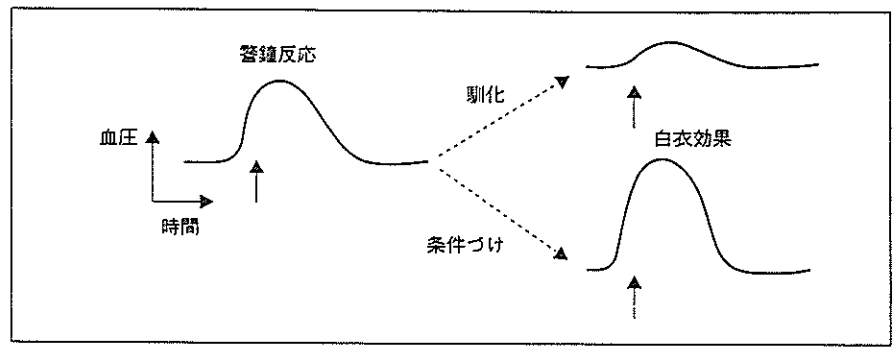
白衣高血圧の診断

白衣高血圧の診断や白衣効果の評価には，家庭血圧測定あるいはABPMが不可欠である。白衣高血圧の特徴は受診時の血圧上昇が継続してみられることであり，診断は繰り返しの血圧測定によりなされねばならず，受診回数とともに血圧が低下する慣れの現象と区別する必要がある。

白衣高血圧の診断基準は確立しているわけではないが，わが国の高血圧治療ガイドライン2004に従えば，外来での随時血圧が140/90mmHg以上で，家庭血圧135/85mmHg未満あるいは24時間血圧135/80mmHg未満となろう。ABPMの日中血圧は24時間平均値より少し高くなるが，米国のガイドライン(JNC 7)は135/85mmHgを高血圧の基準値としている。

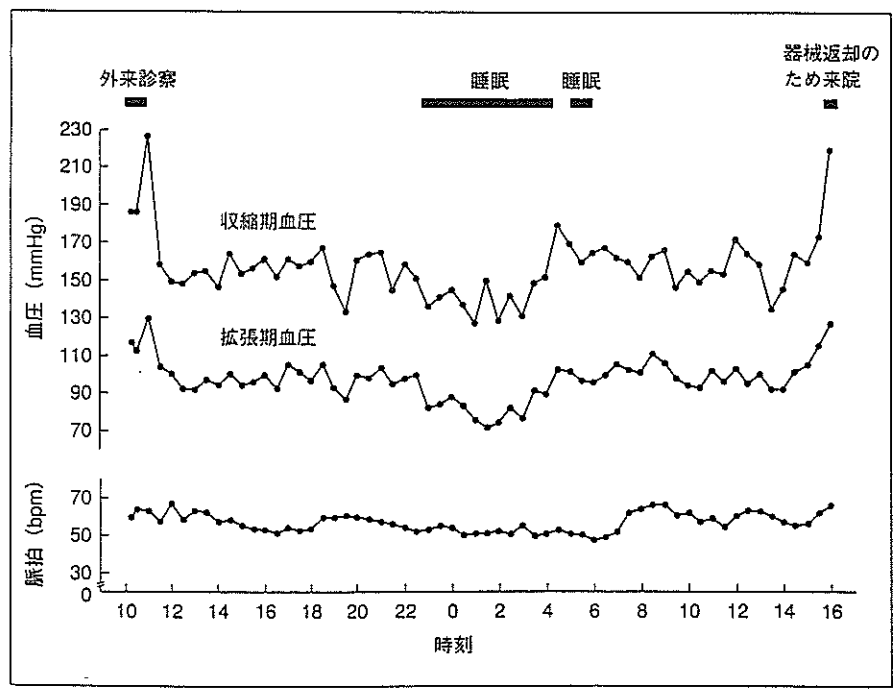
外来患者で随時血圧がかなり高いが高血圧性の臓器障害がない場合には，白衣高血圧の可能性が高い。このような例については，家庭血圧測定が特に勧められる。ABPMは白衣高血圧の確定診断に推奨されているが，通常は家庭血圧による評価で十分であろう。

図3 ▶ 白衣効果の機序



(文献1より引用)

図4 ▶ 著しい白衣効果を示した高血圧症例の自由行動下血圧



(文献7より引用)