*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-$\beta_1$ (*Tgfb1*, nt 1142–1546), rat fibronectin (*Fn1*, nt 619–1082), mouse α1(IV) collagen (*Col4a1*, nt 5808–6165) and mouse TGF-$\beta_1$ (*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

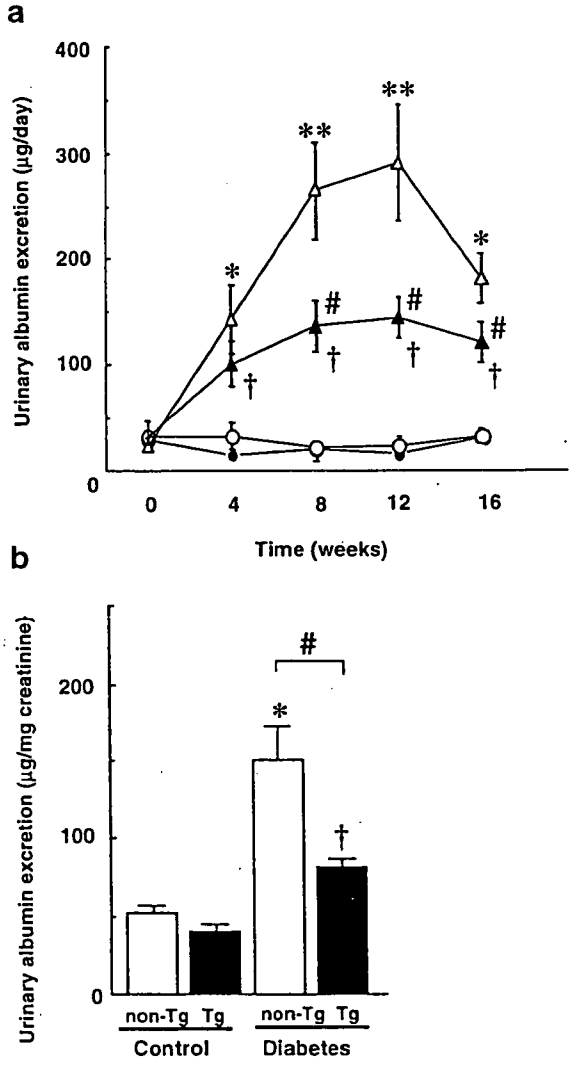


**Fig. 1** a Daily urinary albumin excretion of control and diabetic mice at 0, 4, 8, 12 and 16 weeks after induction of diabetes. *Open circles*, control non-Tg mice ($n$=5); *closed circles*, control BNP-Tg mice ($n$=8); *open triangles*, diabetic non-Tg mice ($n$=10); *closed triangles*, diabetic 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




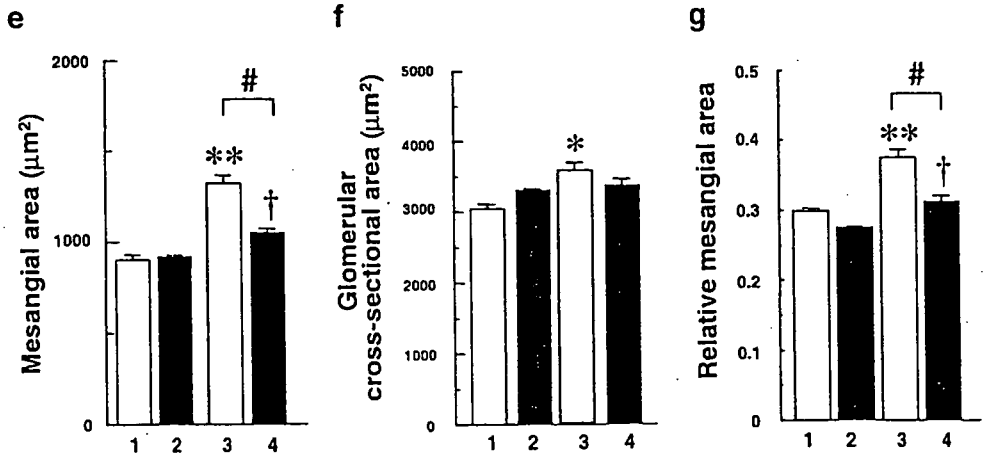


Fig. 2 Glomerular histology of diabetic mice at 16 weeks after induction of diabetes. Representative glomeruli from control non-Tg (a), control BNP-Tg (b), diabetic non-Tg (c), and diabetic BNP-Tg (d) mice are shown. Periodic acid–Schiff stain; magnification: ×400. Glomerular mesangial area (e), glomerular 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

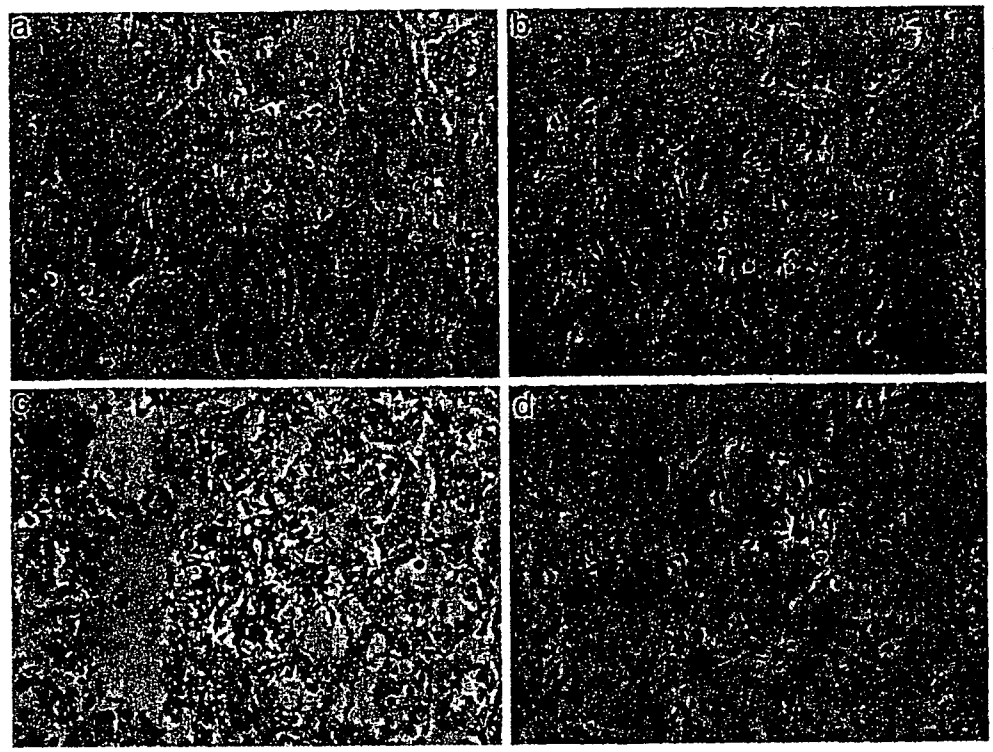


Fig. 3 Immunohistochemistry of TGF-β in control and diabetic mouse kidney at 16 weeks after induction of diabetes. Representative glomeruli from control non-Tg (**a**), control BNP-Tg (**b**), diabetic non-Tg (**c**), and diabetic BNP-Tg (**d**) mice are shown. Magnification: ×400

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 $\mu m^2$, $p<0.01$, $n=10$ 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 $\mu m^2$, $p<0.05$), but was not apparent in BNP-Tg mice (3,378±103 vs 3,313± 30 $\mu m^2$ 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-$\beta_1$ (*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).

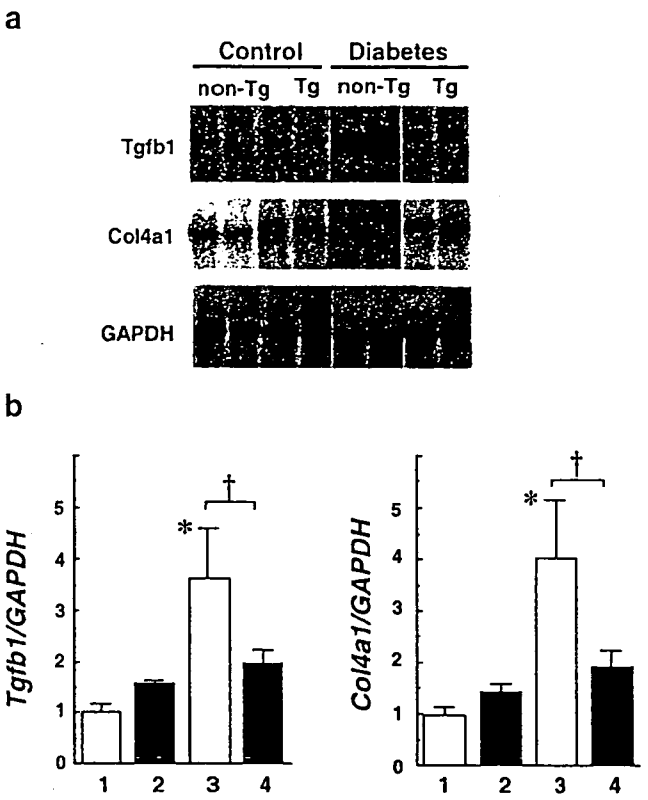


Fig. 4 Representative northern blots for *Tgfb1* and *Col4a1* mRNA expression (a) and their quantitative analysis (b) in kidney of control and diabetic mice at 16 weeks after induction of diabetes. 1, control non-Tg mice ($n$=4); 2, control BNP-Tg mice ($n$=4); 3, diabetic non-Tg mice ($n$=7); 4, diabetic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

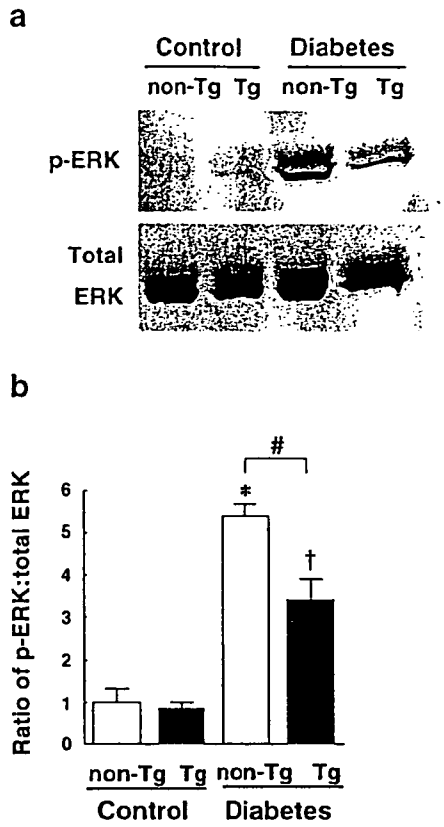


Fig. 5 ERK phosphorylation in the kidney of diabetic mice. Representative western blots of phosphorylated ERK-1/2 and total ERK-1/2 at 16 weeks after induction of diabetes (a) and quantitative analysis of phospho-ERK:total ERK (b). Values are expressed as means±SEM for $n$=4 in each group. *$p$<0.01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

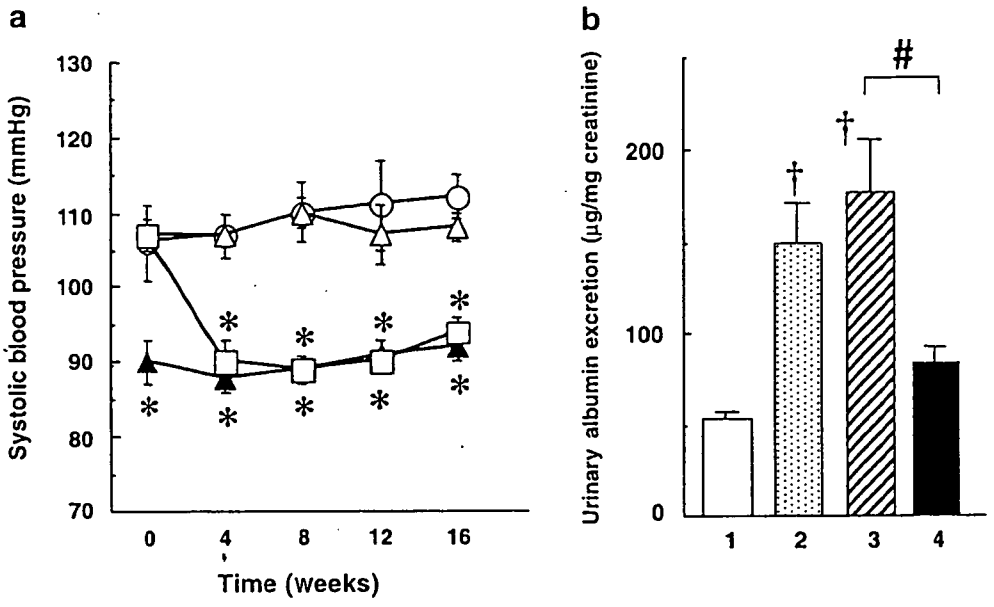


**Fig. 6** a Effects of hydralazine administration on systolic blood pressure. *Open circles*, control non-Tg mice ($n$=5); *open triangles*, diabetic non-Tg mice ($n$=10); *closed triangles*, diabetic BNP-Tg mice ($n$=10); *open squares*, hydralazine-treated diabetic non-Tg mice ($n$=7). *$p<0.005$ 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).

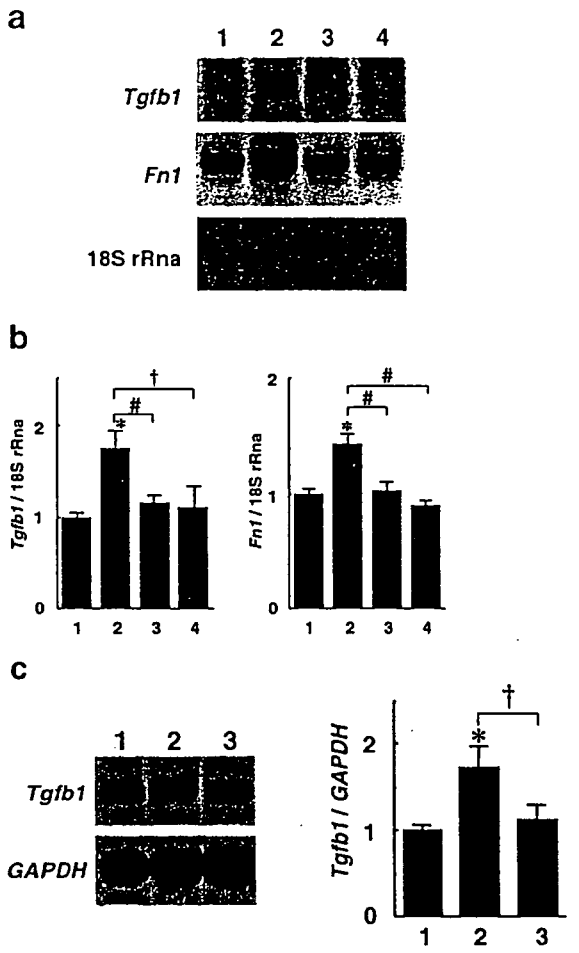


Fig. 7 Representative northern blots for *Tgfb1* and *Fn1* mRNA expression (a) and their quantitative analysis (b) in cultured mesangial cells under high glucose conditions. 1, normal glucose (5.6 mmol/l); 2, high glucose (25 mmol/l); 3, high glucose+BNP 100 nmol/l; 4, high glucose+cGMP 1 mmol/l. Values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

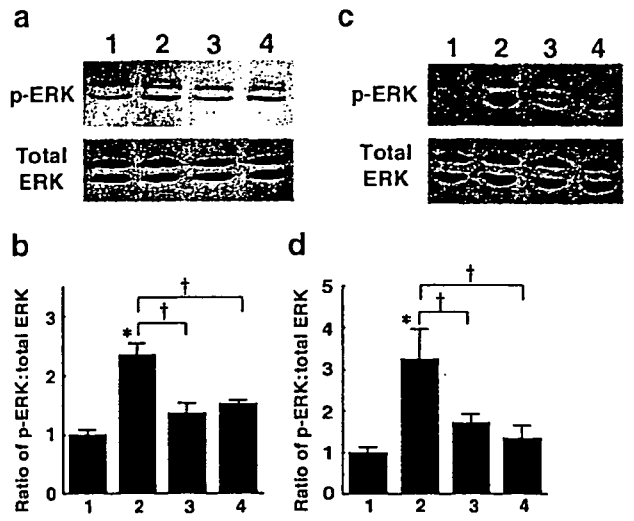


Fig. 8 a, b Inhibitory effects of BNP on ERK phosphorylation in cultured mesangial cells under high glucose conditions. 1, normal glucose (5.6 mmol/l); 2, high glucose (25 mmol/l); 3, high glucose+ BNP 100 nmol/l; 4, high glucose+cGMP 1 mmol/l. Values are expressed as means±SEM for $n$=4 in each group. *$p$<0.01 vs normal glucose; †$p$<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; †$p$<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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# Masked Hypertension and Target Organ Damage in Treated Hypertensive Patients

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**Background:** Recent studies have shown that an elevated ambulatory or home blood pressure (BP) in the absence of office BP—a phenomenon called masked hypertension—is associated with poor cardiovascular prognosis. However, it remains to be elucidated how masked hypertension modifies target organ damage in treated hypertensive patients.

**Methods:** A total of 332 outpatients with chronically treated essential hypertension were enrolled in the present study. Patients were classified into four groups according to office (<140/90 or ≥140/90 mm Hg) and daytime ambulatory (<135/85 or ≥135/85 mm Hg) BP levels; ie, controlled hypertension (low office and ambulatory BP), white-coat hypertension (high office but low ambulatory BP), masked hypertension (low office but high ambulatory BP), and sustained hypertension (high office and ambulatory BP). Left ventricular mass index, carotid maximal intima-media thickness, and urinary albumin levels were determined in all subjects.

**Results:** Of the patients, 51 (15%), 65 (20%), 74 (22%), and 142 (43%) were identified as having controlled hypertension, white-coat hypertension, masked hypertension, and sustained hypertension, respectively. Left ventricular mass index, maximal intima-media thickness, and urinary albumin level in masked hypertension were significantly higher than in controlled hypertension and white-coat hypertension, and were similar to those in sustained hypertension. Multivariate regression analyses revealed that the presence of masked hypertension was one of the independent determinants of left ventricular hypertrophy, carotid atherosclerosis, and albuminuria.

**Conclusions:** Our findings indicate that masked hypertension is associated with advanced target organ damage in treated hypertensive patients, comparable to that in cases of sustained hypertension. Am J Hypertens 2006; 19:880–886 © 2006 American Journal of Hypertension, Ltd.

**Key Words:** Blood pressure, ambulatory, cardiac hypertrophy, atherosclerosis, albuminuria.

Several population-based studies and prospective clinical studies have shown that ambulatory blood pressure (BP) is a significant predictor for cardiovascular morbidity and mortality even after adjustment for conventional BP.[1–3] In fact, left ventricular (LV) hypertrophy and other end-organ damage are more closely associated with average BP levels assessed by 24-h ambulatory monitoring than isolated BP readings taken in the office.[4,5] There is often a discrepancy between office and ambulatory BP, and many studies have evaluated the association between white-coat hypertension, a normal ambulatory but elevated office BP, and cardiovascular risk.[6,7] On the other hand, the converse of white-coat hypertension called "reverse white-coat hypertension," "white-coat normotension," or "isolated ambulatory hypertension," ie, a high ambulatory but normal office BP, has received little attention. This phenomenon is also called "masked hypertension" on the grounds that the hypertension is not detected by routine methods in the clinic.[8] Recent studies indicated that an elevated ambulatory or home BP despite a normal or well-controlled office BP is associated with poor cardiovascular prognosis in both untreated and treated hypertensive patients.[9–11] The present study was conducted to verify

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the possible association between masked hypertension and target organ abnormalities such as LV hypertrophy, carotid arteriosclerosis, and albuminuria in treated hypertensive patients.

# Methods

## Subjects

A total of 332 outpatients with treated essential hypertension (163 men and 169 women; mean age, 66 ± 10 years) were enrolled in the present study. Patients with secondary hypertension, stroke, ischemic heart disease including myocardial infarction, congestive heart failure, chronic glomerulonephritis, nephrotic syndrome, renal failure (serum creatinine ≥160 μmol/L), or poorly controlled (fasting plasma glucose ≥10.0 mmol/L or hemoglobin $A_{1c}$ ≥8.0%) or insulin-treated diabetes mellitus were excluded from this study. Diabetes mellitus was diagnosed according to the American Diabetes Association criteria, such as a fasting plasma glucose of ≥7.0 mmol/L and/or a plasma glucose level at 2 h after a 75-g oral glucose load of ≥11.1 mmol/L, or when medication was taken for treatment of hyperglycemia. A diagnosis of hyperlipidemia required a serum total cholesterol level of ≥5.69 mmol/L and/or a serum triglyceride level of ≥1.69 mmol/L or the use of lipid-lowering drugs.

All patients had taken antihypertensive drugs for at least 1 year (average, 13 years). Of the patients, 237 patients (71%) were treated with Ca channel blockers, 111 (33%) with angiotensin II receptor blockers, 56 (17%) with angiotensin converting enzyme inhibitors, 106 (32%) with β-blockers, 66 (20%) with diuretics, and 34 (10%) with other classes of agents. Combination drug treatments were used in 188 subjects (57%). All subjects gave their informed consent to participate in the present study. All procedures of the present study were carried out in accordance with institutional and national ethical guidelines for human studies.

## Measurement of BP

In each visit, office BP was measured twice by a physician in a hospital outpatient clinic with the patient in a sitting position after ≥20 min of rest, using an appropriate-sized arm cuff and mercury sphygmomanometer. The first and fifth Korotkoff sounds were used to identify systolic and diastolic values, respectively. Office BP was determined by averaging six measurements taken on three separate occasions during a 3-month period.

In the same study period, all subjects underwent 24-h ambulatory BP monitoring. BP and heart rate were measured every 30 min during the day and night by the oscillometric method using an automatic monitoring device (TM-2421, A&D Co., Tokyo, Japan).[12] Accuracy and performance of this device have been previously demonstrated.[13] The patients were instructed to continue with their normal daily activities during measurements and to note their activity and location in a diary. According to the diary, daytime and night-time were determined as the waking and sleeping periods of the patient, respectively, and mean values of 24-h, daytime, and night-time BP (systolic and diastolic) were calculated. We also analyzed short-term BP variability and circadian BP variation. Short-term BP variability was calculated as the standard deviation (SD) of daytime and night-time ambulatory BP obtained every 30 min. Circadian BP variation was defined as a nocturnal dipping in BP and calculated as 100 × (daytime BP − night-time BP) / daytime BP.

In the present study, all subjects were classified into four groups based on the levels of office and daytime ambulatory BP, as follows: 1) controlled hypertension (ie, office BP <140/90 mm Hg and daytime ambulatory BP <135/85 mm Hg); 2) white-coat hypertension (ie, isolated uncontrolled office hypertension, office BP ≥140/90 mm Hg, and ambulatory BP <135/85 mm Hg); 3) masked hypertension (ie, isolated uncontrolled ambulatory hypertension, office BP <140/90 mm Hg and ambulatory BP ≥135/85 mm Hg); and 4) sustained hypertension (ie, uncontrolled hypertension, office BP ≥140/90 mm Hg and ambulatory BP ≥135/85 mm Hg).

## Echocardiography

A comprehensive two-dimensional echocardiography was performed using a cardiac ultrasound unit (Sonos 5500, Philips Medical Systems, Andover, MA) as previously described.[14] Echocardiographic parameters were measured by the consensus of two experienced investigators who were blinded to the clinical data including office and ambulatory BP of the subjects. Measurements included interventricular septal thickness (IVSTd), posterior wall thickness (PWTd), LV diameter at end-diastole (LVDd), and LV diameter at end-systole (LVDs). The LV mass was estimated using the formula validated by Devereux and Reichek[15]: LV mass (g) = $1.04 \times \{(\text{IVSTd} + \text{PWTd} + \text{LVDd})^3 - \text{LVDd}^3\} - 13.6$. The LV mass was normalized for body surface area and expressed as the LV mass index. The intra- and interobserver coefficients of variation of LV mass index were 6.7% and 9.8%, respectively.

## Carotid Ultrasonography

Ultrasound examinations of both carotid arteries were performed using a high resolution Duplex scanner (model SSA-390A, Toshiba, Tokyo, Japan) with the probe at a frequency of 7.5 MHz for the B-scan, as previously described.[16] All measurements were performed by two trained sonographers who were unaware of the subjects' clinical data. The carotid arteries were carefully examined with regard to wall changes from different longitudinal and transverse views. The common carotid artery, the carotid bulb, and the internal and external carotid arteries were studied in all subjects. Each ultrasound image was taken at the end-diastolic phase. We assessed carotid intima-media thickness (IMT) and plaques by measuring generally used

parameters such as conventional IMT and maximal IMT.[16] Conventional IMT was defined as an average of six IMT approximately 15 mm proximal to the carotid bulb in the right and left common carotid arteries avoiding discrete plaques. Maximal IMT was defined as the maximal thickness of intima-media including plaques. Maximal IMT was assessed from the region branching off from the brachiocephalic artery (right) or aorta (left) to the bifurcation of the common carotid artery. The intra- and inter-observer coefficients of variation of maximal IMT were 4.2% and 7.9%, respectively.

## Biochemical Measurements

Blood samples were obtained in the morning after an overnight fast. Total cholesterol, triglycerides, fasting plasma glucose, hemoglobin $A_{1c}$, fasting insulin, and serum creatinine levels were determined by standard laboratory measurements. The homeostasis model assessment (HOMA) index, a parameter of insulin resistance, was calculated as fasting plasma glucose × fasting insulin/22.5. Creatinine clearance was calculated from the Cockcroft-Gault formula.[17] The urinary albumin (U-Alb) level was measured as the ratio of albumin to creatinine excretion in the urine and expressed as $\log_{10}$ mg/g Cr.

## Statistical Analysis

Statistical analysis was performed using StatView Version 5 Software (Abacus Concepts, Berkeley, CA). Values are expressed as the mean ± SD. The significance of differences among the four groups with controlled, white-coat, masked, and sustained hypertension was evaluated by unpaired ANOVA with subsequent Fisher's multiple comparison test. A stepwise multiple regression analysis was performed to identify independent determinants of target organ damage (LV mass index, maximal IMT, and U-Alb levels). A value of $P < .05$ was accepted as statistically significant.

# Results

General characteristics of the four subject groups classified according to certain levels of office BP (<140/90 or ≥140/90 mm Hg) and daytime ambulatory BP (<135/85 or ≥135/85 mm Hg) are summarized in Table 1. Of the patients, 51 (15%), 65 (20%), 74 (22%), and 142 (43%) were identified as having controlled hypertension, white-coat hypertension, masked hypertension, and sustained hypertension, respectively. Age was youngest and the proportion of men was highest in subjects with masked hypertension. The rate of habitual drinkers was significantly

**Table 1.** Clinical characteristics and antihypertensive treatment of study patients

| Characteristic | Controlled hypertension ($n$ = 51) | White-coat hypertension ($n$ = 65) | Masked hypertension ($n$ = 74) | Sustained hypertension ($n$ = 142) |
|---|---|---|---|---|
| Age (y) | 67 ± 8 | 67 ± 7 | 63 ± 11*† | 67 ± 10‡ |
| Sex (male/female) | 24/27 | 23/42 | 48/26*† | 68/74‡ |
| Body mass index (kg/m$^2$) | 24 ± 3 | 24 ± 3 | 25 ± 4 | 24 ± 3 |
| Duration of hypertension (y) | 19 ± 11 | 20 ± 11 | 17 ± 10 | 18 ± 11 |
| Diabetes mellitus (%) | 14 | 23 | 20 | 21 |
| Hyperlipidemia (%) | 59 | 72 | 70 | 67 |
| Current smoking (%) | 16 | 17 | 20 | 18 |
| Habitual drinking (%) | 51 | 46 | 66† | 52 |
| Total cholesterol (mmol/L) | 5.1 ± 0.6 | 5.3 ± 0.8 | 5.3 ± 0.8 | 5.3 ± 0.7 |
| Triglycerides (mmol/L) | 1.4 ± 0.6 | 1.3 ± 0.8 | 1.6 ± 0.9† | 1.5 ± 0.9 |
| Fasting plasma glucose (mmol/L) | 5.7 ± 1.6 | 5.7 ± 1.2 | 5.7 ± 1.0 | 5.8 ± 1.0 |
| Hemoglobin $A_{1c}$ (%) | 5.5 ± 0.7 | 5.7 ± 0.9 | 5.5 ± 0.6 | 5.7 ± 0.8 |
| Fasting insulin (mU/L) | 6.0 ± 2.6 | 6.7 ± 4.3 | 7.4 ± 4.5 | 7.7 ± 11.7 |
| HOMA index | 1.5 ± 0.9 | 1.8 ± 1.5 | 1.9 ± 1.2 | 2.0 ± 3.1 |
| Creatinine clearance (mL/min) | 81 ± 26 | 81 ± 23 | 89 ± 36 | 80 ± 26 |
| Antihypertensive treatment | | | | |
| Period of medication (y) | 13 ± 9 | 14 ± 10 | 11 ± 9† | 13 ± 9 |
| Ca channel blockers (%) | 57 | 74* | 76* | 73* |
| AII receptor blockers (%) | 41 | 28 | 38 | 31 |
| ACE inhibitors (%) | 18 | 12 | 15 | 20 |
| β-Blockers (%) | 31 | 45 | 31 | 27† |
| Diuretics (%) | 20 | 23 | 27 | 15‡ |
| Others (%) | 16 | 5 | 8 | 12 |
| Combination treatment (%) | 55 | 62 | 68 | 49‡ |
| Total number of classes | 1.8 ± 1.0 | 1.9 ± 0.8 | 1.9 ± 0.8 | 1.8 ± 1.0 |

ACE = angiotensin-converting enzyme; AII = angiotensin II; HOMA = homeostasis model assessment.
Values are mean ± SD or percentage.
* $P < .05$ $v$ controlled hypertension; † $P < .05$ $v$ white-coat hypertension; ‡ $P < .05$ $v$ masked hypertension.

increased in masked hypertension compared with white-coat hypertension. Body mass index, duration of hypertension, the prevalence of diabetes mellitus and hyperlipidemia, and the rate of current smokers did not differ among the four groups. In addition, there were no intergroup differences in metabolic parameters and renal function, except that triglyceride level was somewhat increased in masked hypertension.

As for antihypertensive treatment, the period of medication was significantly shorter in masked hypertension than in white-coat hypertension, probably reflecting that the mean age of the group with masked hypertension was lowest. The percentage of the use of Ca channel blockers was significantly higher in white-coat hypertension, masked hypertension, and sustained hypertension than in controlled hypertension. The percentage of treatment with β-blockers or diuretics and that of combination treatment were lower in sustained hypertension than in white-coat hypertension or masked hypertension. The use of angiotensin II receptor antagonists or angiotensin-converting enzyme inhibitors and total number of classes of antihypertensive drugs did not differ among the four groups.

As shown in Table 2, clear differences in office and ambulatory BP levels were observed among the groups with controlled, white-coat, masked, and sustained hypertension. The standard deviations of ambulatory daytime and night-time BP values were significantly increased in masked hypertension compared with controlled hypertension or white-coat hypertension. The degree of nocturnal dipping in systolic BP was significantly larger in masked hypertension than in controlled and white-coat hypertension.

The LV and carotid arterial structural changes and U-Alb levels in the four groups are shown in Fig. 1. The LV mass index ($g/m^2$) was significantly increased in masked hypertension (134 ± 29) than in controlled hypertension (115 ± 34) and white-coat hypertension (119 ± 32). Its level in sustained hypertension (126 ± 32) was significantly higher only compared with that in controlled hypertension. There was no difference in conventional IMT among the four groups (data not shown). However, maximal IMT (mm), which more sensitively reflects the severity of carotid atherosclerosis than conventional IMT,[16] was significantly greater in masked hypertension (1.93 ± 1.07) than in controlled hypertension (1.61 ± 0.67) and white-coat hypertension (1.60 ± 0.82). The level in sustained hypertension (1.69 ± 0.92) was not significantly higher compared with those in controlled and white-coat hypertension. The patients with masked hypertension tended to have more increased LV mass index and maximal IMT than those with sustained hypertension ($P < .10$, respectively). The U-Alb levels ($log_{10}$ mg/g Cr) were significantly higher in masked (1.43 ± 0.62) and sustained hypertension (1.42 ± 0.55) than in controlled (1.12 ± 0.43) and white-coat hypertension (1.22 ± 0.47), and the

**Table 2.** Office and ambulatory blood pressure (BP), heart rate, and BP variability in study patients

| Characteristic | Controlled hypertension ($n = 51$) | White-coat hypertension ($n = 65$) | Masked hypertension ($n = 74$) | Sustained hypertension ($n = 142$) |
|---|---|---|---|---|
| Office BP (mm Hg) | | | | |
| Systolic | 130 ± 6 | 152 ± 16* | 130 ± 6† | 155 ± 15*‡ |
| Diastolic | 75 ± 8 | 86 ± 12* | 77 ± 7† | 88 ± 11*‡ |
| Ambulatory BP (mm Hg) | | | | |
| 24-h Systolic | 125 ± 7 | 128 ± 9 | 137 ± 8*† | 144 ± 13*†‡ |
| 24-h Diastolic | 72 ± 7 | 74 ± 7 | 82 ± 8*† | 83 ± 10*† |
| Daytime systolic | 127 ± 6 | 129 ± 6 | 142 ± 7*† | 147 ± 14*†‡ |
| Daytime diastolic | 73 ± 6 | 76 ± 7 | 85 ± 9*† | 86 ± 11*† |
| Night time systolic | 120 ± 11 | 121 ± 12 | 129 ± 13*† | 137 ± 16*†‡ |
| Night time diastolic | 68 ± 8 | 70 ± 9 | 77 ± 9*† | 78 ± 11*† |
| Heart rate (beats/min) | | | | |
| Office | 67 ± 10 | 69 ± 9 | 68 ± 9 | 70 ± 9* |
| 24-h | 64 ± 9 | 63 ± 11 | 68 ± 9*† | 68 ± 10*† |
| Daytime | 67 ± 10 | 66 ± 10 | 70 ± 10† | 70 ± 11*† |
| Night time | 58 ± 8 | 59 ± 9 | 62 ± 9*† | 62 ± 9*† |
| SD of ambulatory BP (mm Hg) | | | | |
| Daytime systolic | 14.2 ± 3.5 | 13.7 ± 2.8 | 15.0 ± 3.8† | 14.7 ± 4.6 |
| Daytime diastolic | 9.9 ± 3.1 | 9.5 ± 2.7 | 10.8 ± 3.4*† | 10.1 ± 2.9 |
| Night time systolic | 11.6 ± 3.4 | 10.8 ± 3.7 | 12.0 ± 3.9† | 11.4 ± 3.3 |
| Night time diastolic | 8.6 ± 2.3 | 7.9 ± 2.8 | 9.0 ± 2.5† | 8.5 ± 2.4 |
| Nocturnal BP dipping (%) | | | | |
| Systolic | 5.6 ± 8.1 | 5.6 ± 8.3 | 8.7 ± 7.7*† | 6.7 ± 8.6 |
| Diastolic | 7.0 ± 8.6 | 7.6 ± 8.4 | 9.9 ± 7.7 | 8.4 ± 8.7 |

Values are mean ± SD.
* $P < .05$ *v* controlled hypertension; † $P < .05$ *v* white-coat hypertension; ‡ $P < .05$ *v* masked hypertension.

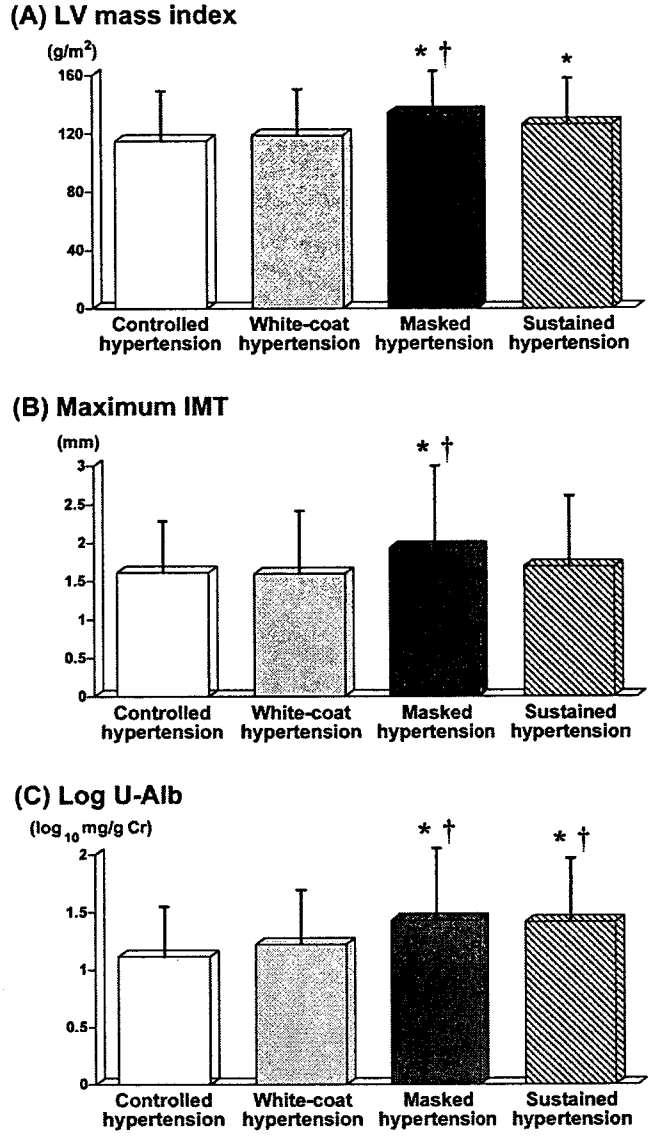


**FIG. 1.** Left ventricular (LV) mass index (**A**), maximal intima-media thickness (IMT) (**B**), and urinary albumin (U-Alb) (log scale, C) in the four study groups, divided by office and daytime ambulatory blood pressure levels. Values are given as mean ± SD. *$P < .05$ *v* controlled hypertension; †$P < .05$ *v* white-coat hypertension.

levels in masked hypertension and sustained hypertension were almost the same.

To identify independent predictors for target organ changes, we investigated possible determination factors using a stepwise multiple regression analysis in all subjects. Although daytime systolic BP was a strong predictor for LV mass index, maximal IMT, and U-Alb levels, the presence of masked hypertension was found to be one of the independent determinants of these end-organ changes (Table 3).

## Discussion

There have been a few studies reporting the possible association between masked hypertension and cardiac and carotid arterial structural changes in the general population. In a cross-sectional study, Liu et al[18] found that LV mass and carotid wall thickness in patients with masked hypertension were significantly greater than those in true normotensive subjects and similar to those in patients with sustained hypertension. The data from the Pressione Arteriose Monitorate E Loro Associazioni (PAMELA) Study also showed that LV mass index was increased in untreated subjects with isolated ambulatory hypertension and sustained hypertension than in those with true normotension.[19] The present findings were broadly consistent with these previous observations. Therefore, our study suggests that a higher level of ambulatory BP largely affects target organ damage in treated hypertensive patients as well as in untreated subjects. In the present study, however, the average levels of 24-h, daytime, and night-time ambulatory BP in the masked hypertension group were somewhat lower than those in the sustained hypertension group. In addition, the presence of masked hypertension was a significant predictor for end-organ changes, independent of average daytime BP levels. Thus factors other than a higher ambulatory BP could contribute to target organ damage in masked hypertension. A shorter period of antihypertensive medication might partially explain the advanced target organ changes in patients with masked hypertension.

In the present study, 22% of subjects were identified as having masked hypertension, which was associated with a higher proportion of men and younger age. These characteristics observed in our study are in agreement with those of masked hypertension described in other studies.[20,21] Increased physical and mental activities in younger men are likely to induce the augmentation of daytime BP variability, which might promote cardiac, carotid arterial, and renal damage in masked hypertension, because several studies have shown that short-term BP variability, apart from average ambulatory BP values, is associated with target organ damage in hypertensive patients.[22–25]

Two recent large-scale prospective studies revealed that a high ambulatory or home BP is a powerful predictor for cardiovascular morbidity in patients with treated hypertension even when their office BP is well controlled. One study by Clement et al[10] showed that the relative risk of cardiovascular events associated with a high 24-h ambulatory systolic BP (≥135 mm Hg) as compared with a low 24-h systolic BP (<135 mm Hg) was 3.19 (unadjusted) or 2.80 (after adjustment) among patients with an office systolic BP of <140 mm Hg. In another cohort study by Bobrie et al,[11] the incidences of cardiovascular events in patients with controlled hypertension (office BP <140/90 mm Hg and home BP <135/85 mm Hg), elevated BP in the office but not at home (ie, white-coat hypertension), elevated BP at home but not in the office (ie, masked hypertension), and uncontrolled hypertension (ie, sustained hypertension) were 11.1, 12.1, 30.6, and 25.6 cases per 1000 patient-years, respectively. The hazard ratio of cardiovascular events in the group with masked hyperten-

**Table 3.** Independent predictors for target organ damage by multivariate regression analysis

| Characteristic | β-Coefficient | F value | P value |
|---|---|---|---|
| LV mass index | | | |
| Daytime systolic BP | 0.270 | 25.64 | <.0001 |
| Sex (male) | 0.190 | 13.53 | .0002 |
| Presence of masked hypertension | 0.136 | 6.56 | .0101 |
| Daytime heart rate | −0.135 | 6.25 | .0112 |
| Duration of hypertension | 0.119 | 5.69 | .0164 |
| Body mass index | 0.110 | 5.22 | .0268 |
| SD of daytime systolic BP | 0.102 | 4.76 | .0476 |
| | $R^2 = 0.240, F = 14.37, P < .0001$ | | |
| Maximum IMT | | | |
| Age | 0.342 | 37.47 | <.0001 |
| Daytime systolic BP | 0.233 | 14.91 | .0001 |
| Daytime diastolic BP | −0.252 | 12.51 | .0006 |
| Sex (male) | 0.184 | 11.38 | .0008 |
| Presence of masked hypertension | 0.157 | 10.04 | .0025 |
| Current smoking | 0.120 | 6.09 | .0225 |
| | $R^2 = 0.275, F = 12.42, P < .0001$ | | |
| Log U-Alb | | | |
| Daytime systolic BP | 0.237 | 18.38 | <.0001 |
| Use of Ca channel blocker | 0.166 | 8.84 | .0035 |
| Creatinine clearance | −0.168 | 8.82 | .0030 |
| Period of antihypertensive medication | 0.159 | 7.16 | .0067 |
| Presence of diabetes mellitus | 0.126 | 5.08 | .0232 |
| Presence of masked hypertension | 0.114 | 4.02 | .0421 |
| | $R^2 = 0.205, F = 11.77, P < .0001$ | | |

BP = blood pressure; Ca = calcium; IMT = intima-media thickness; LV = left ventricular; SD = standard deviation; U-Alb = urinary albumin.
The stepwise regression model included age, sex, body mass index, duration of hypertension, diabetes mellitus, hyperlipidemia, current smoking, habitual drinking, creatinine clearance, period of antihypertensive medication, use of each class of antihypertensive drug (Ca channel blocker, angiotensin II receptor blocker, angiotensin converting enzyme inhibitor, β-blocker, or diuretic), daytime systolic BP, daytime diastolic BP, daytime heart rate, SD of daytime systolic BP, SD of daytime diastolic BP, white-coat hypertension, masked hypertension, and sustained hypertension, as possible independent variables.

sion was shown to be greatest among the four subgroups by an analysis with the multivariable Cox model. Interestingly, our present findings were consistent with these observations examining the prognostic significance of masked hypertension in treated hypertensive subjects. Therefore, the progression of end-organ damage induced by masked hypertension may lead to the high incidence of cardiovascular events in such patients.

There were some limitations in our study. The sample size of our subjects might be relatively small to evaluate properly the differences in target organ damage among the four groups of patients. In addition, the present findings were derived from cross-sectional data on the basis of one-time examination of ambulatory BP monitoring, cardiac and carotid ultrasonography, and urinalysis. Thus a prospective study using larger population of hypertensive subjects will be required to confirm the influence of masked hypertension on target organ damage.

All patients in the present study had received antihypertensive medication. As another limitation of this study, we must therefore consider the possibility that different classes of antihypertensive drugs may have differently affected the development of target organ damage, partly independently of their BP-lowering effects. Renin-angiotensin system inhibitors, above all, have been known to have BP fall–independent protective effects on hypertensive target organ, although the percentage of patients treated with angiotensin II receptor antagonists or angiotensin converting enzyme inhibitors did not differ among the four study groups. Our multivariate analysis showed that the association of masked hypertension with target organ damage was independent of the use of any class of antihypertensive agent. However, approximately 60% of the present subjects were under combination drug treatments. In those cases, the possible specific effect of one or another class of antihypertensive drug could hardly be account for.

In conclusion, the present study shows that masked hypertension is associated with increased LV mass, carotid IMT, and albuminuria in patients with treated essential hypertension, and that the impact of masked hypertension on such end-organ changes is greater than that of controlled hypertension or white-coat hypertension and comparable to that of sustained hypertension. Masked hypertension as well as uncontrolled hypertension is a significant risk for target organ damage in treated hypertensive patients and ambulatory BP monitoring seems to be necessary to unmask this latent risk that is not detectable by routine BP measuring in the office.

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*Original Article*

# Genetic Variations of *HSD11B2* in Hypertensive Patients and in the General Population, Six Rare Missense/Frameshift Mutations

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**Mutations in the gene encoding 11β-hydroxysteroid dehydrogenase type 2, *HSD11B2*, cause a rare monogenic juvenile hypertensive syndrome called apparent mineralocorticoid excess (AME). In AME, defective HSD11B2 enzyme activity results in overstimulation of the mineralocorticoid receptor (MR) by cortisol, causing sodium retention, hypokalemia, and salt-dependent hypertension. Here, we have studied whether genetic variations in *HDS11B2* are implicated in essential hypertension in Japanese hypertensives and the general population. By sequencing the entire coding region and the promoter region of *HDS11B2* in 953 Japanese hypertensives, we identified five missense mutations in 11 patients (L14F, *n*=5; R74H, *n*=1; R147H, *n*=3; T156I, *n*=1; R335H, *n*=1) and one novel frameshift mutation (4884Gdel, *n*=1) in a heterozygous state, in addition to 19 genetic variations. All genetic variations identified were rare, with minor allele frequencies less than 0.005. Four of 12 patients with the missense/frameshift mutations showed renal failure. Four missense mutations, L14F, R74H, R147H, and R335H, were successfully genotyped in the general population, with a sample size of 3,655 individuals (2,175 normotensives and 1,480 hypertensives). Mutations L14F, R74H, R147H, and R335H were identified in hypertensives (*n*=6, 8, 3, and 0, respectively) and normotensives (*n*=8, 12, 5, and 0, respectively) with a similar frequency, suggesting that these missense mutations may not strongly affect the etiology of essential hypertension. Since the allele frequency of all of the genetic variations identified in this study was rare, an association study was not conducted. Taken together, our results indicate that missense mutations in *HSD11B2* do not substantially contribute to essential hypertension in Japanese. (*Hypertens Res* 2006; 29: 243–252)**

***Key Words*: HSD11B2, missense mutation, genetic variation, essential hypertension, salt-sensitivity**

## Introduction

In mineralocorticoid target organs, the 11β-hydroxysteroid dehydrogenase (HSD11B) catalyzes the interconversion of the endogenous cortisol and cortisone in humans. Two distinct forms, HSD11B1 and HSD11B2, of HSD11B have been characterized and cloned (*1–3*). HSD11B1 is expressed in most tissues. In contrast, HSD11B2 has been identified in a limited range of tissues, such as the distal tubules of the kid-

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ney (*2*, *4*, *5*). In mineralocorticoid-responsive cells, HSD11B2 converts cortisol to cortisone, which is not a ligand for the mineralocorticoid receptor, permitting aldosterone to occupy the receptor.

Apparent mineralocorticoid excess syndrome (AME) is an autosomal recessive disorder that results in severe low-renin hypertension and other characteristic clinical features (*6–8*). Typical patients present with severe hypertension, hypokalemia, and undetectable aldosterone. Most patients also have low birth weight, polyuria and polydipsia, failure to thrive, and nephrocalcinosis. The syndrome has been associated with sudden fatality. The HSD11B2 deficiency has been demonstrated in patients with AME and explains the pathogenesis of the disease, which results from excess cortisol binding to the mineralocorticoid receptor due to a failure to convert cortisol to cortisone (*9–11*). Over the last two decades, various genetic mutations in the *HSD11B2* gene have been reported (*12–17*). In Japanese patients with AME, two missense mutations (S180F, R208H) and a deletion of 3 nucleotides resulting in R337H and delta Y338 have been identified (*14*, *18*).

In 1998, a mild form of this disease characterized by P227L mutation in the *HSD11B2* gene was reported (*19*). In contrast to the patients with AME, this patient had low-renin hypertension and hypoaldosteronism but no other phenotypic features that would lead to the diagnosis of AME. Afterwards, it was reported that the defective allele frequency in a cohort of Mennonites was 1.7% (*20*). The genetic mutation in the *HSD11B2* gene, which results in a mild HSD11B2 deficiency, may represent an important cause of low-renin hypertension, the diagnostic basis of which is mostly unknown. Together, these findings suggest that, because 40% of patients with essential hypertension have low renin, these patients may have a mild form of AME.

In the *HSD11B2* gene, the 535G>A polymorphism (synonymous mutation at E178) in exon 3, which can be distinguished by *Alu* I cleavage and the polymorphic microsatellite marker (*21*), have been reported. The minor allele frequency of the 553G>A polymorphism was 0.086 in a healthy Caucasian population and 0.180 in a group of renal transplant patients ($n$=61), indicating association of this polymorphism with end-stage renal disease. This polymorphism was not associated with essential hypertension (*22*). As for the microsatellite marker, a total of 12 alleles were detected. The urinary ratio of cortisol to cortisone metabolites was higher in subjects homozygous for the A7 microsatellite allele than in the corresponding control subjects. Thus, the association of a polymorphic microsatellite marker of the *HSD11B2* gene with reduced HSD11B2 activity suggests that variants of the *HSD11B2* gene contribute to enhanced blood pressure response to salt in humans (*23*). The study demonstrated that a salt-induced blood pressure increase is associated with impaired HSD11B2 activity, as measured by the urinary excretion ratio of cortisol to cortisone metabolites in young Caucasian salt-sensitive men.

The present study was undertaken 1) to identify the genetic variants in the *HSD11B2* gene in Japanese hypertensives, 2) to address whether individuals with heterozygous missense/frameshift mutations show hypertension or renal impairment, and 3) to explain the genetic contribution to a mild form of hypertension including low-renin hypertension and hypoaldosteronism. We sequenced the promoter and exon regions of *HSD11B2* in Japanese hypertensives and genotyped the rare missense/frameshift mutations in the general population. We assessed the role of these genetic variations in hypertension and clarified their contribution to hypertension in Japanese.

**Table 1. General Characteristics of Patients with Hypertension**

| | |
|---|---|
| Number | 953 |
| Age (years) | 65.1±10.5 |
| Gender (M/F) | 522/431 |
| Body mass index (kg/m$^2$) | 24.2±3.3 |
| SBP (mmHg) | 145.5±19.2 |
| DBP (mmHg) | 84.8±13.4 |
| Essential hypertension | 880 |
| Secondary hypertension | 73 |
| Renal hypertension | 36 |
| Renovascular hypertension | 23 |
| Primary aldosteronism | 11 |
| Hypothyroid-induced hypertension | 2 |
| Renal impairment/failure* | 110 |
| Ischemic heart disease | 102 |
| Stroke | 145 |

Values are expressed as mean±SD. *Serum creatinine ≥1.4 mg/dl. M, male; F, female; SBP, systolic blood pressure; DBP, diastolic blood pressure.

## Methods

### Hypertensive Patients

A total of 953 hypertensive patients (522 men and 431 woman; average age: 65.0±10.5 years) were recruited from the Division of Hypertension and Nephrology at the National Cardiovascular Center as reported previously (*24–27*). Briefly, 92% of study subjects (880 subjects) were diagnosed with essential hypertension, and the rest had secondary hypertension (Table 1). Hypertension was defined as systolic blood pressure (SBP) of ≥140 mmHg, and/or diastolic blood pressure (DBP) of ≥90 mmHg, or current use of antihypertensive medication. Hyperlipidemia was defined by total cholesterol ≥220 mg/dl or current use of antihyperlipidemia medication. Diabetes mellitus was defined by fasting plasma glucose ≥126 mg/dl or HbA1c ≥6.5% or current use of anti-diabetic medication. Study subjects had routine laboratory tests including electrolytes, renal function, blood glucose, HbA1c, plasma renin activity and plasma aldosterone concentration.

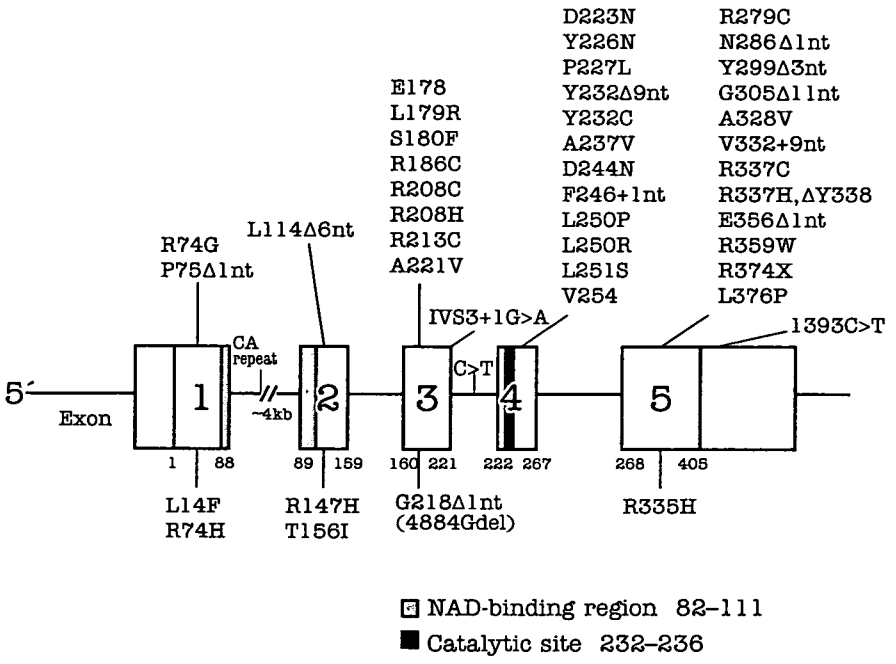


**Fig. 1.** *Summary of the reported genetic polymorphisms in* HSD11B2. *All polymorphisms in the upper section were reported previously, and the six polymorphisms in the lower section were identified in present study.*

## Sequencing of the *HSD11B2* Gene

We sequenced all exons and the promoter region of *HSD11B2* in 953 Japanese hypertensive patients. Blood samples were obtained from hypertensive patients and genomic DNA was isolated from peripheral blood leukocytes. All exons with their flanking sequences and about 1.6 kb of the upstream region were directly sequenced with an ABI PRISM 3700 DNA analyzer (Applied Biosystems, Foster City, USA) using seven sets of primers, as described previously (*28*). Information on the primers and polymerase chain reaction (PCR) conditions is available on request. The obtained sequences were examined for the presence of variations using Sequencher software (Gene Codes Corporation, Ann Arbor, USA), followed by visual inspection. The A of the ATG of the initiator Met codon is denoted as nucleotide +1. The nucleotide sequence (GenBank Accession ID: NT_010498) was used as a reference sequence.

## General Population (the Suita Study)

The sample selection and study design of the Suita Study have been described previously (*29, 30*). Briefly, the subjects visited the National Cardiovascular Center every 2 years for general health checkups. In addition to performing a routine blood examination that included lipid profiles, glucose levels, blood pressure, anthropometric measurements, a physician or nurse administered questionnaires covering personal history of cardiovascular diseases, including angina pectoris, myocardial infarction, and/or stroke. Blood pressure was measured after at least 10 min of rest in a sitting position. SBP and DBP were means of two measurements performed by well-trained doctors using a mercury sphygmomanometer (with a 3-min interval). The subjects were classified as current drinkers if they drank at least 30 ml ethanol per day, nondrinkers if they had never drunk, and past drinkers if they previously had drunk above 30 ml ethanol per day.

## Genotyping of Genetic Variations in the General Population

Genotyping was attempted for six rare missense/frameshift mutations using the TaqMan-PCR method (*31*). The sequences of PCR primers and probes for the TaqMan-PCR method are available on request. Genotyping for two of the six rare mutations—4582C>T (encoded T156I) and 4884Gdel (a frameshift mutation)—failed. Thus, four genetic variations were successfully genotyped in 3,655 participants (1,709 men and 1,946 women) of the large cohort known as the Suita Study. All of the participants for genetic analysis in the present study gave their written informed consent. All clinical data and sequencing and genotyping results were anonymous. The study protocol was approved by the Ethical Review Committee of the National Cardiovascular Center.

**Table 2. Sequence Variations in the Promoter Region and All Exons in *HSD11β2* Identified in Approximately 953 Japanese Patients with Hypertension and/or Renal Failure**

| SNP name | Region | Amino acid substitution | Allele 1 freq. | Allele 2 freq. | Flanking sequence | Genotyping |
|---|---|---|---|---|---|---|
| −879C>T | promoter | | 0.999 | 0.001 | TCCTCTGACA[C/T]CCCACCCTCC | |
| −687C>A | promoter | | 0.999 | 0.001 | CAGGGGTGAG[C/A]GCGCCTTAGG | |
| −596 to −595 CGGCAGins | promoter | | 0.999 | 0.001 | GCAGCGGCAG[CGGCAG]CGGAGACCGG | |
| −562G>T | promoter | | 0.999 | 0.001 | TGGTTCCTCG[G/T]GGTGTTCCTG | |
| −74C>G | promoter | | 0.999 | 0.001 | ACTCCGCGCC[C/G]CGGCCTAGAA | |
| 40C>T | exon 1 | L14F | 0.997 | 0.003 | CGCCTGGCTG[C/T]TCGTGGCTGC | done |
| 42C>A | exon 1 | L14L | 0.999 | 0.001 | CCTGGCTGCT[C/A]GTGGCTGCCC | |
| 82C>T | exon 1 | L28L | 0.999 | 0.001 | GCGCTCAGAC[C/T]TGCGTCTGGG | |
| 221G>A | exon 1 | R74H | 0.999 | 0.001 | CGCCTGGCGC[G/A]CCCGCAGCGC | done |
| 4554G>A | exon 2 | R147H | 0.999 | 0.001 | GACATTAGCC[G/A]CGTGCTAGAG | done |
| 4582C>T | exon 2 | T156I | 1.000 | 0.000 | AAGGCCCACA[C/T]CACCAGCACC | failed |
| 4681G>A | intron 2 | | 1.000 | 0.000 | GCTGACCTAA[G/A]GCTTCCCTCC | |
| 4884Gdel | exon 3 | frame shift | 1.000 | 0.000 | TGACTGTGGG[G]AGCCCAGCGG | failed |
| 4910C>G | intron 3 | | 0.995 | 0.005 | TGCCCCCCCC[C/G]ACTGGAGCAA | |
| 4902insC(8–10) | intron 3 | | 0.998 | 0.002 | GCCCCCCCCC[C]ACTGGAGCAA | |
| 4964C>G | intron 3 | | 0.999 | 0.001 | GAGCCCCTTG[C/G]CAAAGCTGAG | |
| 5017G>A | exon 4 | P227P | 0.997 | 0.003 | TGCCATATCC[G/A]TGCTTGGGGG | |
| 5205G>A | intron 4 | | 0.999 | 0.001 | TATGGGGGCA[G/A]GTCAGGTTTG | |
| 5267G>A | intron 4 | | 0.999 | 0.001 | CAGACCTGGC[G/A]CGGGTTAAAC | |
| 5334C>T | intron 4 | | 0.999 | 0.001 | GCCACTCCTT[C/T]CCCAGAGTCA | |
| 5422C>T | exon 5 | Y295Y | 1.000 | 0.000 | TGCAGGCCTA[C/T]GGCAAGGACT | |
| 5541G>A | exon 5 | R335H | 1.000 | 0.000 | GCTCGGCCCC[G/A]CCGCCGCTAT | done |
| 5698G>A | exon 5 | Q387Q | 1.000 | 0.000 | CCCCACCACA[G/A]GACGCAGCCC | |
| 5759A>G | 3′-UTR | | 1.000 | 0.000 | TCGGTGAGCC[A/G]TGTGCACCTA | |
| 5784C>T | 3′-UTR | | 0.996 | 0.004 | CCAGCCACTG[C/T]AGCACAGGAG | |

The A of the ATG of the initiator Met codon is denoted nucleotide +1, as recommended by the Nomenclature Working Group (*37*). The nucleotide sequence (GenBank Accession ID: NT_010498) was used as a reference sequence. UTR, untranslated region; freq., frequency. Missense mutations were genotyped for general population except two mutations of which genotypes were not determined.

## Results

### Identification of Genetic Variations in *HSD11B2* in a Japanese Hypertensive Population

We sequenced the promoter and exon regions of *HSD11B2* in 953 hypertensives. As a result, we did not identify the reported common genetic variations in Caucasians and causative genetic variations of AME in the *HSD11B2* gene. Instead, we identified five novel missense mutations and one frameshift mutation in *HSD11B2* (Fig. 1, Table 2). Five patients had a C-to-T substitution at nucleotide 40 in exon 1, which led to an amino acid substitution from L to F at position 14 (L14F). One patient had a G-to-A substitution at nucleotide 221 in exon 1, resulting in an amino acid substitution from R to H at position 74 (R74H). Three patients had a G-to-A substitution at nucleotide 4554 in exon 2, leading to an amino acid substitution from R to H at position 147 (R147H). One patient had a C-to-T substitution at nucleotide 4582 in exon 2, leading to an amino acid substitution from T to I at position 156 (T156I). One patient had a G-to-A substitution at nucleotide 5541 in exon 5, resulting in an amino acid substitution from R to H at position 335 (R335H). We also found one patient with a frameshift mutation that resulted from a guanine deletion at position 4884 in exon 3 (4884Gdel). These missense/frameshift mutations were all found in the heterozygous form.

We also identified five synonymous polymorphisms, which encoded for L14 (42C>A in exon 1) with a minor allele frequency of 0.001%, L28 (82C>T in exon 1) with a minor allele frequency of 0.001%, P227 (5017G>A in exon 4) with a minor allele frequency of 0.003%, Y295 (5422C>T in exon 5) with a minor allele frequency of 0.0003% and Q387 (5698G>A in exon 5) with a minor allele frequency of 0.0003%. Fourteen additional genetic variations in the promoter, intronic, and 3′-untranslated regions were also identified. All of the genetic variations were rare, with minor allele frequencies less than 0.005 (Table 2).