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Activity of the Chinese prescription Hachimi-jio-gan against renal damage in the Otsuka Long-Evans Tokushima Fatty rat: a model of human type 2 diabetes mellitus

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Abstract

Currently, in Japan, approximately 95% of patients with diabetes mellitus have non-insulin-dependent (type 2) diabetes mellitus (NIDDM), and diabetic nephropathy is a major cause of patients requiring chronic haemodialysis. A previous study showed that Hachimi-jio-gan has a protective effect in rats subjected to subtotal nephrectomy plus streptozotocin injection, a model of insulin-dependent (type 1) diabetic nephropathy. In this study, we used the Otsuka Long-Evans Tokushima Fatty (OLETF) rat, a model of human NIDDM, to investigate whether long-term administration of Hachimi-jio-gan affects glycaemic control and renal function in NIDDM. Male OLETF rats, aged 22 weeks, were divided into 4 groups of 10 and given Hachimi-jio-gan (50, 100 or 200 mg kg^{-1} daily) orally or no treatment for 32 weeks. Male Long-Evans Tokushima Otsuka (LETO) rats ($n = 6$) were used as non-diabetic normal controls. Hachimi-jio-gan reduced hyperglycaemia dose-dependently from 16 weeks of the administration period. Urinary protein excretion decreased significantly from an early stage, and creatinine clearance levels improved at 32 weeks. In addition, the levels of serum glycosylated protein and renal advanced glycation end-products were effectively reduced. Hachimi-jio-gan also significantly reduced the levels of thiobarbituric acid-reactive substances in renal mitochondria, although it showed only a tendency to reduce these in serum. Furthermore, long-term administration of Hachimi-jio-gan reduced renal cortical expression of proteins, such as transforming growth factor-β_1 (TGF-β_1), fibronectin, inducible nitric oxide synthase and cyclooxygenase-2. The 100- and 200-mg kg^{-1} daily doses of Hachimi-jio-gan significantly reduced TGF-β_1 and fibronectin protein expression to levels below those of LETO rats. These data suggest that Hachimi-jio-gan may have a beneficial effect on the progression of diabetic nephropathy in OLETF rats by attenuating glucose toxicity and renal damage.

Introduction

Renal damage is one of the serious complications of diabetes mellitus. Recently, the number of diabetic patients in Japan has reached seven million with a remarkable increase in non-insulin-dependent (type 2) diabetes mellitus (NIDDM). Furthermore, diabetic nephropathy has been the major cause of patients needing chronic haemodialysis since 1998 (Nakai et al 2004). Prolonged dialysis is a great burden to the patients, both mentally and physically, and social problems, including financial issues, also arise. Therefore, prevention of the onset or progression of diabetic nephropathy is considered to be of the utmost importance to reduce the number of patients with end-stage renal failure.

Diabetic nephropathy is characterized by albuminuria, hypertension, a decline of the glomerular filtration rate (GFR) and glomerular sclerosis (Remuzzi & Bertani 1998). During its development, glucose exerts toxic actions as a result of processes that are activated within the diabetic kidney (e.g., accumulation of advanced glycation end-products, oxidative stress, abnormal polyol metabolism and synthesis of growth factors (Lehmann & Schleicher 2000)). Currently, the basic treatment for diabetic nephropathy is strict control of blood glucose levels and blood pressure and dietary or protein restriction. Clinical and experimental studies have provided evidence

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of various beneficial effects of angiotensin-converting enzyme (ACE) inhibitors, angiotensin II receptor blockers and antihypertensive drugs against diabetic nephropathy (Heart Outcomes Prevention Evaluation (HOPE) Study Investigators 2000; Brenner et al 2001; Lewis et al 2001).

In Japan, the Chinese prescription Hachimi-jio-gan has been traditionally and widely used for the treatment of patients with diabetes mellitus, hypertension, nephrotic syndrome and glomerulonephritis. Experimentally, it has exhibited beneficial effects in animal models with diabetes induced by alloxan (Nagayoshi et al 1966) and streptozotocin (STZ) (Kim et al 2004), and it reduced insulin resistance in a model of NIDDM (Furuya et al 1999). In addition, Hachimi-jio-gan has been reported to have a renoprotective effect in experimental rats with diabetic nephropathy induced by subtotal nephrectomy followed by STZ injection (Nakagawa et al 2001; Yokozawa et al 2004). However the mechanisms responsible for the effects of Hachimi-jio-gan against NIDDM are still unclear.

Otsuka Long-Evans Tokushima Fatty (OLETF) rats, which spontaneously develop type 2 diabetes mellitus and its complications, show the following similar clinical and pathological features to those seen in diabetic nephropathy in man (Kawano et al 1992): late-onset hyperglycaemia associated with mild and chronic disease; mild obesity; hypoinsulinaemia (> 40 weeks of age) and renal complications (i.e., mesangial proliferation and thickening of basement membranes, diffuse glomerular lesions and nodular lesions). We have used this model to investigate the effects of the Chinese prescription Hachimi-jio-gan on glucose metabolism and renal damage in OLETF rats.

Materials and Methods

Materials

The following reagents were purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan): 4,6-dihydroxy-2-mercaptopyrimidine (2-thiobarbituric acid (TBA)); 5-hydroxymethylfurfural (5-HMF); oxalic acid; bovine serum albumin (BSA); 2-amino-2-hydroxymethyl-1,3-propadiol (Tris (hydroxymethyl) aminomethane); NP-40; glycerol; phenylmethyl sulfonyl fluoride (PMSF) and skimmed-milk powder. Protease inhibitor cocktail was purchased from Roche Diagnostics GmbH (Mannheim, Germany) and 4–20% w/v Tris-glycine gel and polyvinylidine difluoride (PVDF) membrane for the detection of transforming growth factor-β_1 (TGF-β_1) and fibronectin were purchased from Invitrogen Life Technologies (Japan). The Bio-Rad protein assay kit and PVDF membrane were purchased from Bio-Rad Laboratories (Japan) and ATTO Bioscience & Biotechnology (Tokyo, Japan), respectively. The following antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA): rabbit polyclonal anti-human TGF-β_1 (sc-146); rabbit polyclonal anti-human nuclear factor-κB (NF-κB) p65 (sc-109); rabbit polyclonal anti-human inhibitor binding protein κB-α (IκB-α) (sc-371); mouse monoclonal anti-mouse NOS2 (sc-7271) (a primary antibody against inducible nitric oxide synthase (iNOS)); mouse monoclonal anti-human cyclooxygenase-2 (COX-2) (sc-19999) and goat anti-rabbit immunoglobulin (Ig) G horseradish peroxidase (HRP)-conjugated (sc-2004) and goat anti-mouse IgG HRP-conjugated (sc-2005) secondary antibodies. Rabbit anti-human fibronectin polyclonal antibody (A0245) was purchased from DAKO Cytomation (Denmark), anti-mouse β-actin antibody was purchased from Sigma-Aldrich (St Louis, MO, USA) and anti-rabbit Ig, HRP-linked whole antibody, anti-mouse Ig, HRP-linked whole antibody and ECL Western Blotting Detection Reagents were purchased from Amersham Bioscience (Piscataway, NJ, USA).

Preparation of Hachimi-jio-gan extract

The Hachimi-jio-gan extract used in this study was produced by Tsumura Juntendo, Inc. (Tokyo, Japan). The composition of Hachimi-jio-gan was as follows: Rehmanniae Radix (*Rehmannia glutinosa* Libosch. var. *purpurea* Makino) 6 g; Corni Fructus (*Cornus officinalis* Sieb. et Zucc.) 3 g; Dioscoreae Rhizoma (*Dioscorea japonica* Thunb.) 3 g; Alismatis Rhizoma (*Alisma orientale* Juzep.) 3 g; Hoelen (*Poria cocos* Wolf) 3 g; Moutan Cortex (*Paeonia suffruticosa* Andrews) 2.5 g; Cinnamomi Cortex (*Cinnamomum cassia* Blume) 1.0 g and Aconiti Tuber (*Aconitum carmichaeli* Debx) 0.5 g. These eight crude drugs were boiled together gently in ten times their volume of water for 60 min, filtered and the filtrate was spray-dried to obtain the extract at a yield of about 10%, by weight, of the original preparation. To analyse the components of Hachimi-jio-gan, the aqueous extract (0.5 g) was extracted with 20 mL methanol under ultrasonication for 30 min. The solution was filtered through a membrane filter (0.45 μm) and then subjected to analysis by high-performance liquid chromatography (HPLC) using a TSK-GEL ODS-80TS column (ϕ 4.6 × 250 mm; TOSOH, Japan) with an LC $10AV_{vp}$ pump and a SPD-$M10A_{vp}$ absorbance detector. The elution solvents were (A) 0.05 M AcOH–$AcONH_4$ and (B) CH_3CN, and the column was eluted with a linear gradient of, by volume, 90% A and 10% B changing over 60 min to 100% B. The flow rate was 1.0 mL min^{-1} and the effluent from the column was monitored and processed into three-dimensional data by an SPD-M10A array detector. All assigned peaks were identified by comparing their ultraviolet (UV) spectral data with those of co-injected authentic samples using Class LC-10 Version 1.62 software (Shimadzu, Japan). The three-dimensional HPLC profile of Hachimi-jio-gan extract is shown in Figure 1. Morroniside, loganin and paeoniflorin were the major components of Hachimi-jio-gan; penta-*O*-galloylglucose, benzoylmesaconine, cinnamic acid, benzoylpaeoniflorin, cinnamaldehyde and 16-ketoalisol A were also detected.

Animals and treatment

The Guidelines for Animal experimentation, approved by Toyama Medical and Pharmaceutical University,

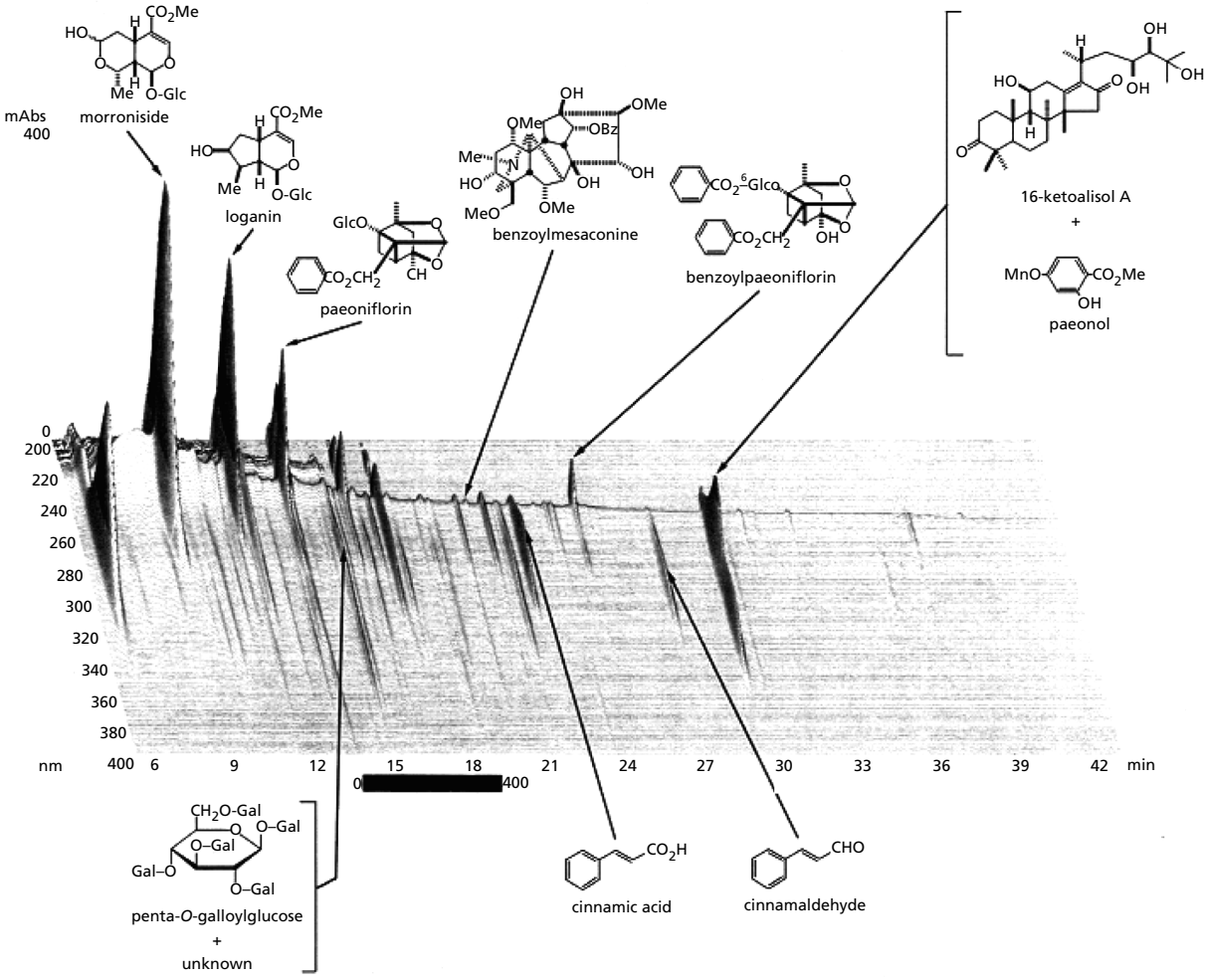

Figure 1 Three-dimensional HPLC profile of Hachimi-jio-gan extract.

were followed in these experiments. Male OLETF and related non-diabetic strain Long-Evans Tokushima Otsuka (LETO) rats were supplied by Otsuka Pharmaceutical Co. Ltd (Tokushima, Japan). All rats were kept in wire-bottomed cages with controlled temperature (about 25°C) and humidity (about 60%) and a 12-h light–dark cycle. Laboratory pellet chow (CE-2, comprising 24.0% protein, 3.5% lipid and 60.5% carbohydrate; CLEA Japan Inc., Tokyo, Japan) and water were freely available. When the rats were aged 22 weeks, we determined blood glucose, urinary protein excretion and creatinine (Cr) clearance levels, and allocated the OLETF rats to four groups of 10 rats, avoiding any significant differences of these levels among the four groups. Over the experimental period of 32 weeks, one diabetic OLETF group and the non-diabetic LETO group received water and the other three OLETF groups received a solution of Hachimi-jio-gan extract (50, 100 or 200 mg kg^{-1} body weight daily) orally by gavage once a day. Every eight weeks, rats were transferred to individual metabolic cages for collection of 24-h urine specimens and blood samples were taken from their tail veins for the determination of blood glucose levels, urinary protein excretion and Cr clearance. At the end of the study, individual 24-h urine specimens were also collected and blood samples were collected by cardiac puncture and serum was immediately separated from the blood samples by centrifugation. After renal perfusion through the renal arteries with ice-cold physiological saline, both kidneys were removed from each rat, rinsed with cold saline, frozen and kept at −80°C until they were assayed.

Determination of serum constituents and urinary protein levels

Serum glucose and Cr levels were measured using commercial kits (Glucose CII-Test Wako obtained from Wako Pure Chemical Industries Ltd (Osaka, Japan) and CRE-EN Kainos obtained from Kainos Laboratories Inc. (Tokyo, Japan), respectively). The extent of serum protein glycosylation was measured by the TBA assay of McFarland et al (1979), in which non-enzymatically bound glucose is released as 5-HMF

and quantitated colorimetrically. In brief, serum samples (100 μL) were diluted to 1.0 mL with distilled water, mixed with 0.5 mL of 1.0 M oxalic acid, hydrolysed for 4.5 h at 100°C and then glycosylated haemoglobin was quantified by measuring the absorbance at 443 nm after reaction with TBA. Serum TBA-reactive substance levels were measured using the method of Naito & Yamanaka (1978). Urinary protein excretion levels were determined by the sulfosalicylic acid method (Sakagishi 1968).

Determination of renal advanced glycation end-products (AGEs) contents

Renal AGEs contents were determined by the method of Nakayama et al (1993). In brief, minced renal tissue was delipidated by shaking gently with chloroform and methanol (2:1, v/v) overnight. After washing with methanol and then distilled water, the pellet was homogenized in 1.0 mL of 0.1 N NaOH and centrifuged at 8000 g for 15 min at 4°C. The amounts of AGEs in these alkali-soluble samples were measured at an emission wavelength of 440 nm and excitation wavelength of 370 nm against a blank of 0.1 N NaOH solution using a spectrofluorometric detector (Shimadzu RF/550; Kyoto, Japan). A native bovine serum albumin (BSA) preparation (1 mg mL^{-1} in 0.1 N NaOH) was used as a reference, and its fluorescence intensity was defined as one unit of fluorescence. The fluorescence intensities of the samples at a protein concentration of 1 mg mL^{-1} were measured and expressed in arbitrary units (AU) relative to the fluorescence intensity of the native BSA preparation.

Preparation of renal mitochondria and determination of their TBA-reactive substance levels

Mitochondria were prepared from renal homogenates by differential centrifugation (800 g and 12 000 g) at 4°C, according to the methods of Johnson & Lardy (1967) and Jung & Pergande (1985), with slight modifications. The pellets were resuspended in preparation medium, and the TBA-reactive substance levels were assayed according to the method of Mihara & Uchiyama (1978). Protein levels were determined by the method of Itzhaki & Gill (1964) with BSA as the standard.

Protein extraction and Western blot analyses

Renal cortical sections were homogenized with ice-cold lysis buffer (pH 7.5) containing 137 mM NaCl, 20 mM Tris-HCl, 1% v/v NP-40, 10% v/v glycerol, 1 mM PMSF and protease inhibitor cocktail (10 μg mL^{-1} aprotinin, 1 μg mL^{-1} leupeptin). Samples were then centrifuged at 2000 g for 10 min at 4°C. To ensure equal loading of the lanes, the protein concentration of each tissue was determined using a Bio-Rad protein assay kit with BSA as the standard and then immunoblotting was carried out.

For determination of TGF-β_1 and fibronectin protein expression, 60 and 10 μg protein, respectively, from each sample was electrophoresed through denaturing 4–20% Tris-glycine gel. The separated proteins were transferred electrophoretically to a PVDF membrane, blocked with 5% skimmed milk solution for 1 h and then incubated with the corresponding primary anti-TGF-β_1, fibronectin or β-actin antibody overnight at 4°C. Then, the blots were washed with Tris-buffered saline containing 0.1% v/v Tween 20, incubated with anti-rabbit and/or anti-mouse Ig HRP-conjugated secondary antibodies for 90 min at room temperature and each antigen–antibody complex was visualized using ECL Western Blotting Detection Reagents and detected by autoradiography with X-ray film.

For determination of iNOS, COX-2, NF-κB p65 and IκB-α protein expression, 30 or 50 μg protein from each sample was electrophoresed through 8 or 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE). Separated proteins were transferred electrophoretically to a PVDF membrane, blocked with 5% skimmed milk solution for 1 h and then incubated with the corresponding primary anti-iNOS, COX-2, NF-κB p65, IκB-α or β-actin antibody overnight at 4°C. Next, the blots were washed with Tris-buffered saline containing 0.1% v/v Tween 20 and incubated with goat anti-rabbit and/or goat anti-mouse IgG HRP-conjugated secondary antibodies for 90 min at room temperature. Each antigen-antibody complex was visualized using ECL Western Blotting Detection Reagents and detected by chemiluminescence with LAS-1000 plus (FUJIFILM, Japan).

Band densities were determined by NIH image software (Scion Corporation, Frederick, MD, USA) and quantified as a ratio of the density of the β-actin band. The mean quantity of each of these proteins from LETO rats is represented as 1 and the corresponding values for the OLETF rats are expressed as ratios of these values.

Statistical analysis

The results are presented as means ± s.e. The effect of Hachimi-jio-gan on each parameter was examined using one-way analysis of variance. Individual differences among groups were analysed by Dunnett's test and those at $P < 0.05$ were accepted as significant.

Results

Body and kidney weight changes

Table 1 shows the changes in body and kidney weights at the end of the study. Untreated OLETF rats weighed significantly more than LETO rats. Hachimi-jio-gan-treated OLETF rats showed a tendency to weigh less than untreated OLETF rats, but the differences were not significant. At the same time, the kidney weight of untreated OLETF rats was also higher than that of

Table 1 Body and kidney weights of LETO rats and untreated and Hachimi-jio-gan-treated OLETF rats

Group	Dose (mg kg^{-1} daily)	Body weight (g)	Kidney weight (g (100 g of body weight)$^{-1}$)
LETO rats	—	558.2 ± 18.0	0.439 ± 0.015
OLETF rats			
Control	—	638.8 ± 17.0[a]	0.505 ± 0.008[a]
Hachimi-jio-gan	50	638.3 ± 13.8[a]	0.518 ± 0.014[a]
Hachimi-jio-gan	100	621.3 ± 14.3[a]	0.535 ± 0.008[a,b]
Hachimi-jio-gan	200	624.1 ± 13.2[a]	0.522 ± 0.010[a]

[a]$P < 0.001$ vs LETO rats; [b]$P < 0.001$ vs OLETF control rats.

LETO rats, whereas no dose of Hachimi-jio-gan affected kidney weight.

Blood glucose levels, urinary protein excretion and Cr clearance

Blood glucose levels during the 32-week experimental period are shown in Figure 2A. LETO rats maintained their blood glucose level at about 145 mg dL^{-1} until 24 weeks, while the levels of the untreated OLETF rats were significantly higher at 182–197 mg dL^{-1} ($P < 0.001$). When Hachimi-jio-gan was administered to OLETF rats, however, a tendency for the elevated glucose level to decrease was apparent at week 16, and at week 24 the magnitude of the increase in the blood glucose level of untreated OLETF rats was reduced significantly in a dose-dependent manner. At the end of this experiment, the blood glucose level of untreated OLETF rats increased further to 236 mg dL^{-1}, but that of Hachimi-jio-gan (200 mg kg^{-1} daily)-treated OLETF rats was significantly lower than this (217 mg dL^{-1}, $P < 0.01$).

Initially, the urinary protein excretion rate of untreated OLETF rats (about 50 mg/day) was more than twice that of LETO rats (22 mg/day) and it gradually increased with age, reaching 130 mg/day by 24 weeks (Figure 2B). At 32 weeks, the value for untreated OLETF rats was significantly (about 17 times) higher (222 mg/day) than that for LETO rats (13 mg/day), indicating marked proteinuria in the former. Hachimi-jio-gan treatment markedly reduced the urinary protein excretion rates from an early stage ($P < 0.001$), and this was maintained up to week 32. In particular, the value for rats treated with Hachimi-jio-gan 200 mg kg^{-1} was about 70% (158 mg/day) of that for untreated OLETF rats.

The Cr clearance of each group fluctuated irregularly until 24 weeks (Figure 2C), but at 32 weeks, it was significantly lower in untreated OLETF than LETO rats (4.6 ± 0.1 and 3.8 ± 0.1 mL kg^{-1} per day, respectively, $P < 0.001$). However, the Cr clearances of OLETF rats treated with 50, 100 and 200 mg kg^{-1} Hachimi-jio-gan daily improved compared with the OLETF rats by 4.2, 4.2 and 4.6 mL kg^{-1} per day, respectively (Figure 2C).

Serum glycosylated protein and TBA-reactive substance levels

The serum glycosylated protein level of OLETF rats was significantly elevated to about 1.7 times the LETO value, whereas oral administration of Hachimi-jio-gan prevented this increase significantly and dose-dependently (Table 2). The TBA-reactive substance level was also significantly increased in OLETF rats (3.58 nmol L^{-1}) compared with LETO rats (2.26 nmol L^{-1}, $P < 0.001$), but Hachimi-jio-gan-treated rats showed a tendency, which was not significant, to decreased levels compared with untreated OLETF rats.

Renal AGEs and TBA-reactive substance levels

The renal levels of AGEs and mitochondrial TBA-reactive substances are shown in Table 3. At 32 weeks, AGEs accumulated in untreated OLETF rats and the level was about 1.2-fold that of LETO rats ($P < 0.001$). The levels of the groups treated with Hachimi-jio-gan were effectively reduced to the normal level by the lowest dose (50 mg kg^{-1} daily), and the reductions were significant and dose-dependent. The renal mitochondrial TBA-reactive substance level of untreated OLETF rats was 1.8-fold that of LETO rats and the 50-, 100- and 200-mg kg^{-1} daily doses of Hachimi-jio-gan reduced it significantly and non-dose-dependently to 80, 76 and 76% that of the untreated OLETF rats, respectively ($P < 0.001$), unlike its minimal effect on the serum TBA-reactive substance level.

Western blot analyses

At 32 weeks, the level of TGF-β_1 (12.5 kDa) and fibronectin (220 kDa) expression in renal cortex were both clearly higher in untreated OLETF rats than in LETO rats (Figure 3A, B, upper panels). Densitometric analysis showed that TGF-β_1 and fibronectin protein levels in untreated OLETF rats were 1.22- and 1.16-fold those of LETO rats, respectively (Figure 3A, B, bottom panels). However, these expression levels in Hachimi-jio-gan-treated rats were both reduced markedly, especially by the 100- and 200-mg kg^{-1} daily doses (respectively: to 71% and 59% of the level in untreated OLETF rats for TGF-β_1 and to 87% and 77% for fibronectin) and these reductions were larger than the difference between untreated OLETF and LETO rats.

Expressed iNOS and COX-2 proteins were detected at 130 kDa and 72 kDa, respectively, and the levels of untreated OLETF rats were several times higher than those of LETO rats (Figure 3C, D). Administration of Hachimi-jio-gan inhibited the expression of iNOS and COX-2 proteins starting at a dose of 100 mg kg^{-1} daily, and significant reductions of 21% ($P < 0.001$) and 9% ($P < 0.01$), respectively, compared with the untreated

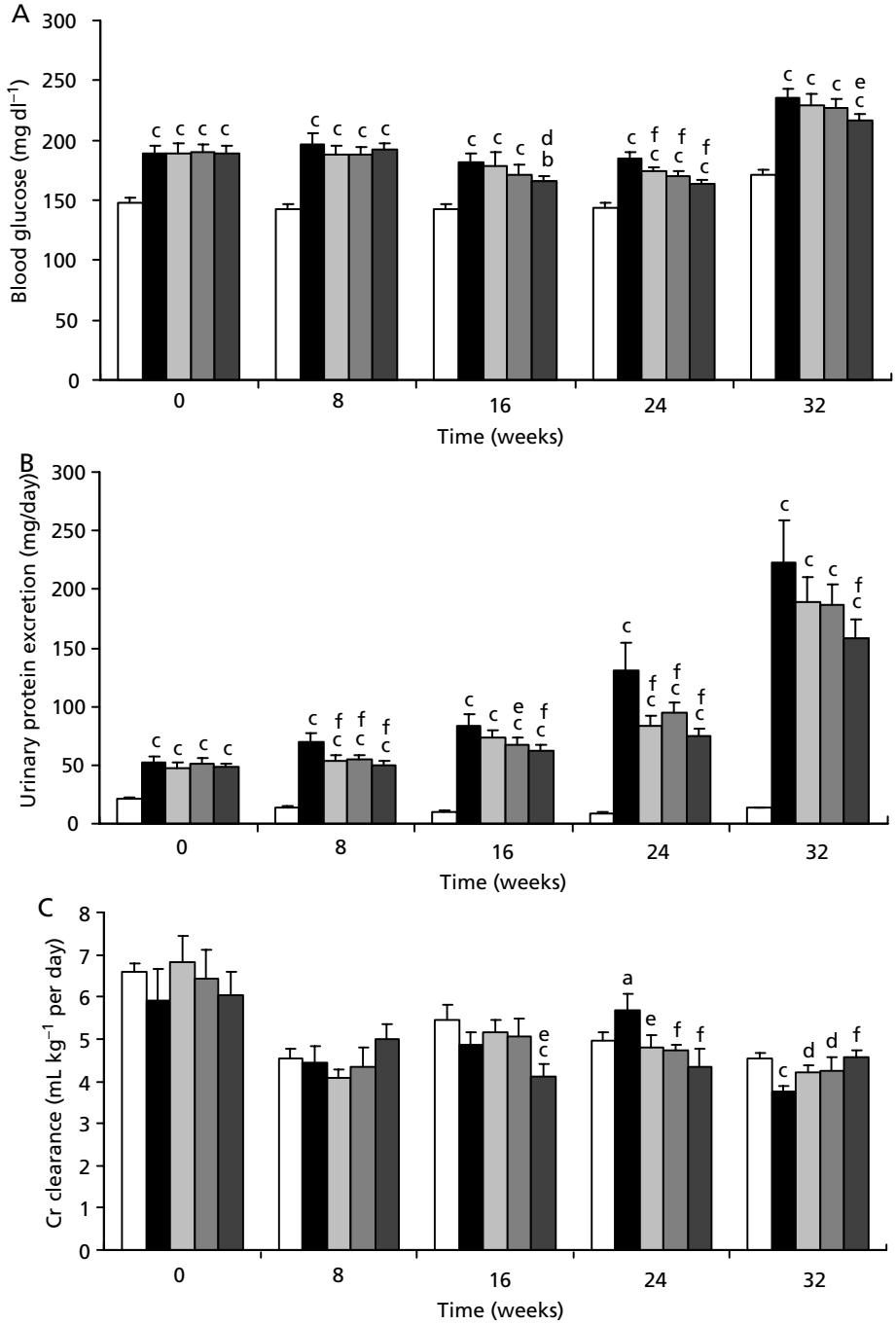

Figure 2 Blood glucose (A), urinary protein (B) and Cr clearance (C) in LETO rats (□) and in OLETF rats treated with either Hachimi-jio-gan (50 mg kg^{-1} daily, (▫) ; 100 mg kg^{-1} daily, (■); 200 mg kg^{-1} daily, (■)) or control (■) for 32 weeks. [a]$P < 0.05$, [b]$P < 0.01$, [c]$P < 0.001$ vs LETO rats; [d]$P < 0.05$, [e]$P < 0.01$, [f]$P < 0.001$ vs untreated OLETF rats.

OLETF group, were observed in OLETF rats given 200 mg kg^{-1} daily.

In addition, we investigated the effects of Hachimi-jio-gan on NF-κB p65 (65 kDa) and IκB-α (37 kDa). There was no significant difference between the NF-κB p65 protein levels of untreated OLETF and LETO rats, whereas a significant increase in the phosphorylated IκB-α protein level (1.12 fold) was observed in the former compared with the latter (Figure 3E, F, upper panels). After long-term administration of Hachimi-jio-gan, however, the NF-κB p65 expression levels were similar to those of the other groups, whereas Hachimi-jio-gan, at doses of 100 and 200 mg kg^{-1} daily, markedly reduced the expression of phosphorylated IκB-α protein (to 93%, $P < 0.05$, and 88%, $P < 0.001$, of the expression in untreated OLETF rats) (Figure 3E, F, bottom panels).

Table 2 Serum glycosylated protein and TBA-reactive substance levels in LETO rats and untreated and Hachimi-jio-gan-treated OLETF rats

Group	Dose (mg kg^{-1} daily)	Glycosylated protein (nmol (mg protein)$^{-1}$)	TBA-reactive substance (nmol L^{-1})
LETO rats	—	11.4 ± 0.6	2.26 ± 0.25
OLETF rats			
Control	—	19.2 ± 1.1^{b}	3.58 ± 0.32^{b}
Hachimi-jio-gan	50	$16.0 \pm 1.1^{b,c}$	3.47 ± 0.20^{b}
Hachimi-jio-gan	100	$15.3 \pm 1.0^{b,c}$	3.39 ± 0.19^{b}
Hachimi-jio-gan	200	$13.4 \pm 0.9^{a,c}$	3.27 ± 0.24^{b}

$^{a}P < 0.05$, $^{b}P < 0.001$ vs LETO rats; $^{c}P < 0.001$ vs OLETF control rats.

Table 3 Renal AGEs and TBA-reactive substance levels in LETO rats and untreated and Hachimi-jio-gan-treated OLETF rats

Group	Dose (mg kg^{-1} daily)	AGEs (AU)	TBA-reactive substance (nmol (mg protein)$^{-1}$)
LETO rats	—	0.75 ± 0.02	2.22 ± 0.24
OLETF rats			
Control	—	0.89 ± 0.03^{b}	3.99 ± 0.19^{b}
Hachimi-jio-gan	50	0.75 ± 0.03^{c}	3.23 ± 0.19^{c}
Hachimi-jio-gan	100	0.73 ± 0.02^{c}	3.04 ± 0.21^{c}
Hachimi-jio-gan	200	$0.68 \pm 0.03^{a,c}$	3.02 ± 0.15^{c}

$^{a}P < 0.01$, $^{b}P < 0.001$ vs LETO rats; $^{c}P < 0.001$ vs OLETF control rats.

Discussion

The aims of this study were to investigate whether Hachimi-jio-gan exhibits renoprotective effects during the development of type 2 diabetic nephropathy. In a previous study, we used an animal model of insulin-dependent (type 1) diabetic nephropathy, rats subjected to subtotal nephrectomy plus injection of STZ, and demonstrated that Hachimi-jio-gan has a protective effect on the kidney through the amelioration of metabolic disorders, oxidative stress and renal dysfunction (Yokozawa et al 2004). We have further studied the effects of Hachimi-jio-gan in spontaneously diabetic WBN/Kob rats, which have insulin sensitivity. Hachimi-jio-gan could prevent diabetic kidney damage by reducing renal oxidative injury and expression of fibronectin and TGF-β_1 proteins, which are all involved in the pathophysiology of diabetic nephropathy (Nakagawa et al 2005). To add to these findings, in this experiment we investigated the effects of Hachimi-jio-gan treatment in the OLETF rat, an established suitable model of type 2 diabetes mellitus.

At 22 weeks of age, OLETF rats had significantly greater body weights, blood glucose levels and proteinuria than LETO rats, which do not develop diabetes mellitus, and from the same age, OLETF rats have been reported to develop the initial histopathological changes of diabetic nephropathy (Fukuzawa et al 1996). In addition, Kawano et al (1992) have described proliferation of the mesangial matrix and thickening of glomerular basement membranes in OLETF rats at 40 weeks of age. Up to now, therapeutic research with this model has demonstrated the effects of thiazolidinediones (Onozaki et al 2004), bezafibrate (Jia et al 2004), ACE inhibitors (Sugimoto et al 2001) and angiotensin-II receptor blockers (Hayashi et al 2003) against diabetes mellitus, its cardiac and renal complications and pancreatic function. Based upon these features, we performed this experiment using OLETF rats, 22–54 weeks old, to clarify the functional mechanisms responsible for the effects of Hachimi-jio-gan during the phase when type 2 diabetic nephropathy develops.

Proteinuria is a key predictor of declining glomerular filtration rate (GFR) in patients with diabetic nephropathy, and occurs as a result of glomerular capillary hypertension and damage to barrier functions in the glomerular basement membrane. Microalbuminuria has been reported to be associated with a 2- to 4-fold increase in the risk of death, and overt proteinuria and hypertension are associated with an even higher risk when present together (Dinneen & Gerstein 1997). However, patients with NIDDM, who are affected by environmental influences (access to high-calorie foods, lack of exercise and weight gain) in addition to genetic influences, have many other risk factors (e.g., ageing, complications due to hypertension and hyperlipidaemia, cardiovascular disease, etc.). Furthermore, in many cases, it is unclear, due to lack of medical examination, exactly when the onset of type 2 diabetes mellitus occurs and also to what extent the nephropathy has progressed in such patients. Therefore, reducing prolonged continual proteinuria may afford protection against the tubulointerstitial injury accompanying glomerular lesions in diabetic nephropathy. At the beginning of this study, OLETF rats already showed a significant, over 2-fold, increase in the urinary protein excretion rate compared with LETO rats, while Hachimi-jio-gan markedly inhibited this increase from the early stages of the administration period. These results are similar to those of our previous study on type 1 diabetic nephropathy (Yokozawa et al 2004), indicating that Hachimi-jio-gan may, at least in part, have effects against renal damage such as glomerular lesions and tubulointerstitial injury.

Conversely, Cr clearance levels, reflecting the GFR, were higher in untreated OLETF rats than LETO or Hachimi-jio-gan-treated OLETF rats at 24 weeks of the administration period, indicating glomerular hyperfiltration, which led to a decline in their renal function at 32 weeks. However, abnormal renal function was normalized by the administration of Hachimi-jio-gan, suggesting that long-term treatment with Hachimi-jio-gan may counteract renal damage and postpone end-stage renal disease.

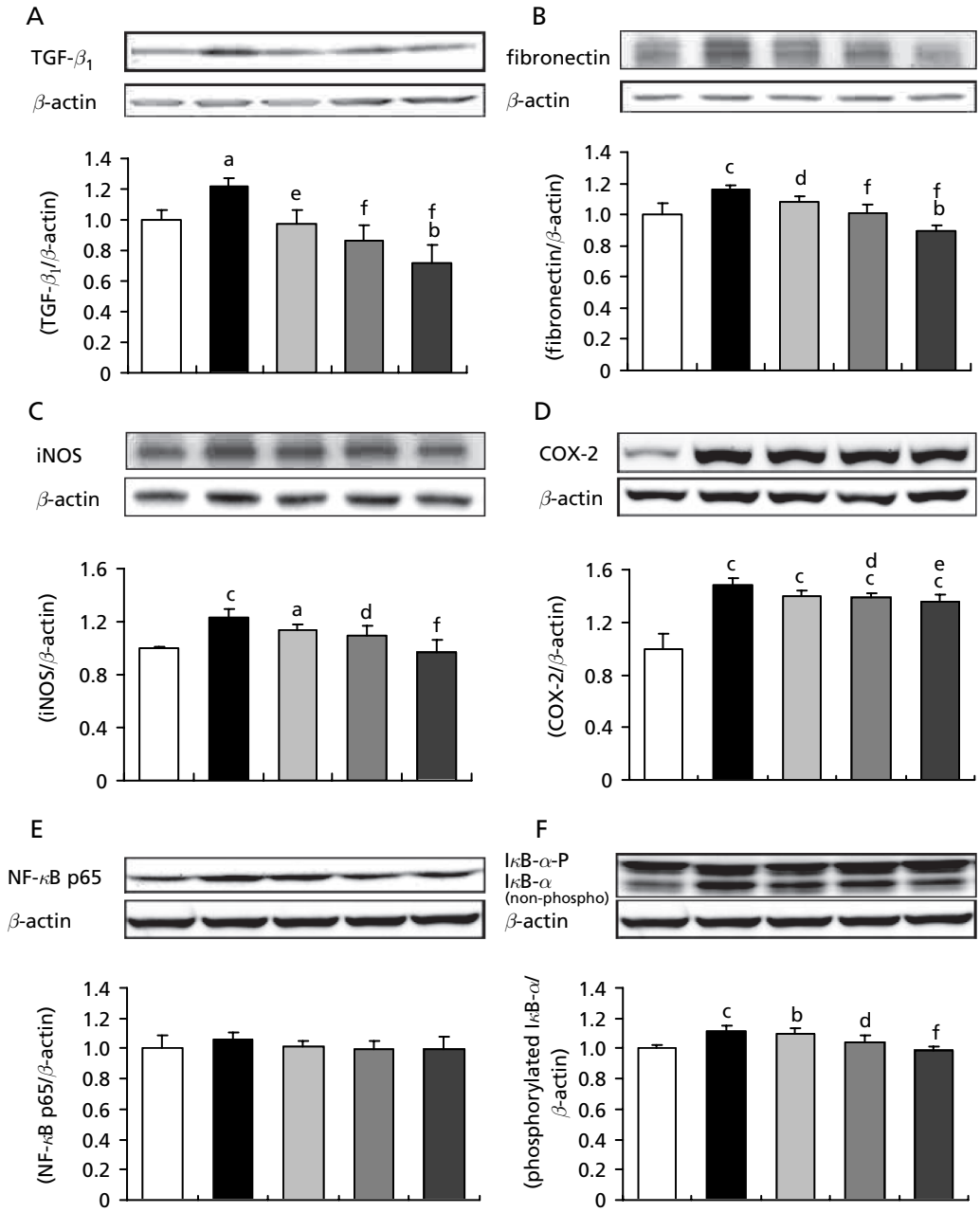

Figure 3 Western blot analyses of TGF-β_1 (A), fibronectin (B), iNOS (C), COX-2 (D), NF-κB p65 (E) and IκB-α (phosphorylated and non-phosphorylated) (F) in renal cortex of LETO rats (□) and in OLETF rats treated with either Hachimi-jio-gan (50 mg kg^{-1} daily, (▩); 100 mg kg^{-1} daily, (▩); 200 mg kg^{-1} daily, (▩)) or control (■) for 32 weeks. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$ vs LETO rats; $^{d}P < 0.05$, $^{e}P < 0.01$, $^{f}P < 0.001$ vs untreated OLETF rats.

The importance of glycaemic control was demonstrated by the 6- and 8-year Kumamoto Study in Japanese patients with type 2 diabetes mellitus (Ohkubo et al 1995; Shichiri et al 2000), in that intensive glycaemic control by multiple insulin injection therapy can effectively delay the onset and the progression of early stages of diabetic microvascular complications, diabetic retinopathy, nephropathy and neuropathy. In addition, hyperglycaemia itself increases lipid peroxidation, an index of increased oxidative stress, in various tissues, including glomerular mesangial cells, and the various factors overlap and interact with one another. In particular, the AGEs seem to be key factors in the pathogenesis of diabetes mellitus and development of diabetic nephropathy, including microvascular and macrovascular injury. That is, plasma proteins modified by AGE precursors bind to AGE receptors (RAGE) on endothelial cells, mesangial cells and macrophages, leading to receptor-mediated generation of reactive oxygen species (ROS), production of growth factors and cytokines, chronic inflammatory responses, and cellular and vascular dysfunction associated with diabetic complications. AGEs–RAGE ligation activates oxidative

stress, p21 ras and downstream targets such as mitogen-activated protein kinases (MAPKs) and leads to the activation of transcriptional factors, such as NF-κB, causing pathological changes in gene expression (Lander et al 1997; Bierhaus et al 2001; Brownlee 2001). It has also been reported that glycoxidation is involved in both the glucose and free-radical theories of ageing, and glycoxidation was proposed to be a source of permanent and cumulative oxidative damage to long-lived proteins, such as collagen, in ageing and diabetes mellitus (Baynes & Thorpe 1999; Nishikawa et al 2000; Jerums et al 2003). Therefore, inhibition of AGEs formation may play an important role in reducing the development of vascular and renal lesions associated with diabetes mellitus or ageing.

To clarify the role of Hachimi-jio-gan on glucose toxicity, we first measured its effects on blood glucose, serum glycosylated protein and renal AGEs levels. Our results showed that Hachimi-jio-gan prevented glucose levels increasing from 16 weeks of the administration period. OLETF rats had significantly elevated serum glycosylated protein and renal AGEs levels, but Hachimi-jio-gan treatment significantly and dose-dependently reduced these, especially renal AGEs levels, which were reduced to below the level of the non-diabetic LETO rats. Hence, Hachimi-jio-gan is applicable to the remedy against type 2 diabetes mellitus and diabetic complications, and therefore, it may also have an effect against the process of ageing.

It is also considered a key factor in diabetes mellitus that glycation products stimulate breakdown of lipids to malondialdehyde, which is a highly toxic by-product formed, in part, by lipid oxidation-derived free radicals (Wolff et al 1991; Slatter et al 2000). Therefore, to show the influence of Hachimi-jio-gan on oxidative stress, we investigated the TBA-reactive substance levels in the serum and kidney. Long-term administration of Hachimi-jio-gan reduced renal TBA-reactive substance levels significantly, despite only showing a tendency, which was not significant, to reduce serum levels. These findings suggest that the effect of Hachimi-jio-gan in reducing oxidative stress caused by the binding activity of AGEs, plasma proteins and RAGE in glomerular mesangial cells or macrophages is superior to its effect against markers of oxidative stress in serum.

In both diabetic rats and human glomeruli, the AGEs–RAGE interaction in mesangial cells activates TGF-β-Smad signalling pathways and subsequently induces synthesis of fibronectin, type IV (1α) collagen and proteoglycan, which are the individual matrix components, through the generation of angiotensin II via overproduction of ROS, and then promotes adhesion to the extracellular matrix, leading to glomerular sclerosis (Ignotz & Massagué 1987; Yamamoto et al 1993; Fukami et al 2004). Furthermore, protein kinase C (PKC), an important mediator of diabetes-induced vascular dysfunction, has been reported to modulate the function of glucose-inducing vascular endothelial growth factor (VEGF) and TGF-β_1 expression. Administration of a specific PKC β inhibitor to rats with STZ-induced diabetes attenuated glomerular hyperfiltration, reduced the urinary albumin excretion rate and decreased expression of TGF-β_1 and various extracellular matrix proteins, such as fibronectin and type IV (1α) collagen (Ishii et al 1996; Koya et al 1997). Lee et al (2003) observed that inhibition of PKC activity effectively blocked high glucose concentration- and hydrogen peroxide (without a high glucose concentration)-induced TGF-β_1 and fibronectin protein expression in mesangial cells, indicating that there is a relationship between PKC and oxidative stress under conditions of hyperglycaemia. In this study, TGF-β_1 and fibronectin protein expression in untreated OLETF rats significantly increased. However, Hachimi-jio-gan down-regulated their expression to levels below those of non-diabetic LETO rats. These findings suggest that Hachimi-jio-gan may ameliorate functional abnormalities in association with the angiotensin II-TGF-β signalling pathway in mesangial cells that is induced by AGEs–RAGE-mediated ROS, which cause fibronectin overexpression in mesangial cells, leading to glomerular sclerosis. It may also ameliorate the expression of TGF-β_1 and fibronectin proteins induced by PKC activation related to oxidative stress. However, the mechanism of action of Hachimi-jio-gan on PKC activity remains to be elucidated.

While hyperglycaemia up-regulates TGF-β_1 and fibronectin protein synthesis, NF-κB and activator protein-1 expression via ROS generation have important roles (Chen et al 2003). In contrast, activated NF-κB induces iNOS and COX-2 expression, the inducible isoforms that have been reported to contribute to cytotoxicity in some cell types. In general, NF-κB is known to be normally sequestered in the NF-κB/IκB complex and resides in the cytoplasm of unstimulated cells, but activation of NF-κB occurs via phosphorylation of its inhibitory subunit, IκB-α. This phosphorylation precedes rapid degradation of IκB, resulting in active NF-κB release and translocation into the nucleus, where it binds to specific κB-binding sites or interacts with other transcription factors, thereby promoting gene transcription (Baeuerle & Baltimore 1988; Schmidt et al 1995; DiDonato et al 1996). The target genes regulated by NF-κB include several immune and inflammatory factors, such as iNOS and COX-2 (Xie et al 1994; Harris & Breyer 2001). Of interest, in this study, Hachimi-jio-gan reduced renal cortical expression of iNOS, COX-2 and phosphorylated IκB-α proteins in OLETF rats. Therefore, chronic treatment with Hachimi-jio-gan may have an ameliorative effect on renal injury during the development of diabetic nephropathy through inhibition of iNOS and COX-2 signalling pathways.

As discussed above, we focused on the toxicity and renal damage caused by glucose during the course of development of type 2 diabetic nephropathy, and demonstrated beneficial effects of the Chinese prescription Hachimi-jio-gan by estimating levels of glucose-induced oxidative stress, AGEs formation, renal protein expression and urinary protein excretion. However, the mechanisms responsible for the effects of Hachimi-jio-gan on glucose toxicity in other internal organs still need to be

discussed. In particular, type 2 diabetes mellitus is closely related to β-cell dysfunction, leading to defects in insulin action and insulin secretion and then exacerbating insulin resistance. Indeed, OLETF rats have been reported to show three stages of pancreatic histological changes: degenerative changes and necrosis of islets (after 12 weeks of age); fibrosis and enlargement of the islets (after 20 weeks of age) and replacement of islets (after 40 weeks of age) (Kawano et al 1992). Therefore, focusing on pancreatic function may provide other clues that will help to clarify the detailed mechanism(s) responsible for the effects of Hachimi-jio-gan against type 2 diabetic nephropathy.

In conclusion, long-term administration of Hachimi-jio-gan can ameliorate the functional and structural features of type 2 diabetic nephropathy, as observed in our previous study in rats with type 1 diabetic nephropathy.

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