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Lignin-derived lignophenols attenuate oxidative and inflammatory damage to the kidney in streptozotocin-induced diabetic rats

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Abstract

This study investigated the effects of lignin-derived lignophenols (LPs) on the oxidative stress and infiltration of macrophages in the kidney of streptozotocin (STZ)-induced diabetic rats. The diabetic rats were divided into four groups with 0%, 0.11%, 0.33% and 1.0% LP diets. The vehicle-injected controls were given a commercial diet. At 5 weeks, superoxide (O_2^-) production, macrophage kinetics, the degree of fibrosis in glomeruli and mRNA expression for monocyte chemoattractant protein-1 (MCP-1) were examined. The NADPH-stimulated O_2^- levels in the kidney of the diabetic rats treated with 1.0% LP were significantly lower than those in untreated diabetic rats. The number of macrophages, levels of MCP-1 mRNA expression and degree of glomerular fibrosis increased in untreated LP and these levels were significantly lower in 1.0%LP-treated rats. The results suggested that LPs suppress the excess oxidative stress, the infiltration and activation of macrophages and the glomerular expansion in STZ-induced diabetic kidneys.

Keywords: *Lignophenols, superoxide, monocyte chemoattractant protein-1, macrophage, fibrosis, diabetic nephropathy*

Introduction

Diabetic nephropathy is the leading cause of end-stage renal disease and the most frequent cause of mortality in patients with diabetes. Oxidative stress mediated by hyperglycaemia-induced generation of reactive oxygen species (ROSs) has been considered a potential factor in the progression of diabetic complications [1,2]. Nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidase has been implicated as the major source of ROSs, particularly superoxides (O_2^-) in the vasculature in response to high levels of glucose and advanced glycation end products [3–5].

In addition to the accumulation of extracellular matrix protein, which results in mesangial expansion and tubulointerstitial fibrosis, the infiltration of macrophages into the glomeruli and tubulointerstitium is also a characteristic pathogenic feature of diabetic nephropathy [6,7]. Moreover, the expression of monocyte chemoattractant protein-1 (MCP-1), which is involved in the infiltration and activation of macrophages, has been thought to play an important role in the fibrogenic process of diabetic nephropathy [2,8]. Thus far, many antioxidants have been shown to attenuate diabetic nephropathy, such as vitamin E, α-lipoic acid and plant polyphenols [9–12].

Lignin is one of the abundant organic substances in the plant kingdom. Although it comprises 20–30% of the global plant biomass, lignin has seldom been utilized because of its resistance to chemical and biological degradation. Recently, lignophenols (LPs), which are derivatives of lignin, have been isolated using a new phase-separation system developed by Funaoka and Fukatsu [13]. Although LPs have been reported to be highly phenolic and highly stable and

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to exhibit antioxidant properties *in vitro* [14], the physiological role of LPs remains unclear. LPs derived from bamboo have been reported to prevent hydrogen peroxide-induced cell death *in vitro* [15]. By using DNA fragment and terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL), we have previously reported that LPs from the beech tree (*Fagus crenata* Blume) suppress copper- and zinc-mediated apoptosis in PC12 cells [16]. However, there is limited information about whether or not an LP-rich diet has any beneficial effects on diabetic nephropathy.

This study aimed to determine whether LPs could play a role in the progression of the diabetic nephropathy in streptozotocin (STZ)-induced diabetic rats. In this study, we have demonstrated that treatment with LPs attenuates the NADH- and NADPH-stimulated production of O_2^- in the kidney and suppresses the increase in the number of infiltrating macrophages and MCP-1 mRNA expression in rats with STZ-induced diabetic nephropathy.

Materials and methods

Preparation of LPs and diets

LPs are composed of a phenol and a concentrated acid, which converts the native lignin into highly phenolic polymers [13] (Figure 1). LPs were prepared by the two-step method established by Funaoka and Fukatsu [13]. In brief, air-dried beech was ground, and *p*-Cresol (10 ml/g wood) in acetone was added to wood flour with stirring. Thereafter, 72% sulphuric acid was added to the mixture. The reaction mixture was separated into organic and aqueous phases. The organic phase was repeatedly rinsed with water until the pH of the solution became neutral. The LPs were decomposed into substances with lower molecular weights by second functional control and heating after alkali treatment (0.5 mol/l sodium hydroxide). After a 48 h incubation period, the sample was washed several times with cold water and then freeze-dried.

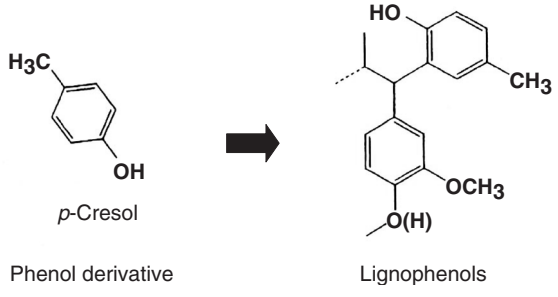

Figure 1. Structural features of the lignophenols derived from lignin by the phase-separation system. The lignophenols were used in this study as mentioned in the materials and methods section.

The molecular weight of the LPs from beech was determined to be ~ 1500.

The LPs were mixed with a standard commercial laboratory diet (MF diet, Oriental Yeast Co. Ltd., Tokyo, Japan). According to the description provided by the manufacturer, the composition of the diet was as follows: 7.7% moisture, 23.6% crude protein, 5.3% crude fat, 6.1% crude ash, 2.9% crude fibre and 54.5% nitrogen-free extract (including carbohydrates), minerals (Ca, 1.12 g; P, 0.90 g; Mg, 0.26 g; Na, 0.21 g; K, 0.99 g/100 g of total minerals; Fe, 10.9 mg; Al, 3.1 mg; Cu, 0.82 mg; Zn, 5.28 mg; Co, 0.10 mg; and Mn, 5.89 mg/100 g of total minerals) and vitamins (retinol, 2160 IU; D_3, 158 IU; E, 11.0 mg; K_3, 0.04 mg; B_1, 2.12 mg; B_2, 1.24 mg; C, 4 mg; B_6, 0.87 mg; B_{12}, 5.3 μg; inositol, 578 mg; biotin, 23.2 μg; pantothenic acid, 2.73 mg; niacin, 10.4 mg; choline, 0.22 g; folic acid, 0.20 mg/100 g of total vitamins).

Animal treatments

All the experimental procedures complied with the regulations of the Guidelines for Animal Experimentation, Aomori University of Health and Welfare. Six weeks-old male Wistar rats (CLEA Japan Inc., Tokyo, Japan) weighing 218–265 g were used. The rats were maintained at a temperature of 23 ± 1°C under a 12 h light–dark cycle starting at 07:00 am. The animals received a single intravenous injection of STZ (65 mg/kg body weight; Sigma Chemical Co., St. Louis, MO) in 0.5 ml of 0.05 mol/l citrate buffer (pH 4.6) into tail vein. Before the start of this experiment, we determined the blood glucose concentrations of all the diabetic rats to be more than 300 mg/dl; these levels were measured 48 h after the STZ injection. The diabetic rats were divided into four groups on the basis of the amount of LPs in their diets: (a) 0% LP (n = 7), (b) 0.11% LP (n = 7), (c) 0.33% LP (n = 8) and (d) 1.0% LP (n = 8). The vehicle-injected control animals (n = 8) were fed the MF diet only. All rats were provided food and tap water *ad libitum*. Their body weights were measured during the LP treatments. At week 4 of this experiment, 24 h urine samples were collected using metabolic cages. The rats were fasted overnight and weighed; their blood samples were collected under ether anaesthesia, following which they were sacrificed at week 5. Their kidneys were quickly extracted, rinsed, weighed and fixed in 4% paraformaldehyde phosphate buffer solution for histopathological and immunohistochemical examinations. Periadventitial tissue of the aorta was carefully extracted. The aorta and the cortices and medullae of the kidneys were stored in chilled saline overnight and used to measure O_2^- production. The other portions of the kidneys were immediately frozen in liquid nitrogen and stored at –80°C for later use.

Blood chemistry

Plasma glucose (Glc) and blood urea nitrogen (BUN) levels were measured using an autoanalyser (Fuji Dry-Chem 3500V, Fujifilm, Tokyo, Japan). Plasma and urinary creatinine (Cr) levels were determined using a commercially available kit (Wako Pure Chemical Industries Ltd., Osaka, Japan).

Determination of 8-hydroxydeoxyguanosine in 24 h urine samples

The levels of 8-hydroxydeoxyguanosine (8-OHdG), a biomarker for oxidative DNA damage, in the 24 h urine samples were determined by enzyme-linked immunosorbent assay (ELISA) by using a commercially available highly sensitive ELISA kit for 8-OHdG (Japan Institute for Control of Aging, Shizuoka, Japan). The urinary concentrations of 8-OHdG per milligram of urinary Cr were calculated.

Determination of superoxide production in the kidney

The cortices and medullae of the extracted kidneys were homogenized in phosphate buffer (pH 7.8) containing Protease Inhibitor Cocktail Set III (Calbiochem, San Diego, CA), centrifuged at 960 *g* for 10 min at 4°C and the supernatant was used for the measurement of O_2^-. Superoxide production in the tissues was determined using a lucigenin (bis-*N*-methyacidinium nitrate)-enhanced chemiluminescence technique [17,18]. The protein content of the supernatant was determined with the BCA™ Protein Assay Kit (Pierce, Rockford, IL). The supernatant (100 μl) was incubated for 5 min in 1 ml lucigenin (5 μmol/l)-phosphate buffer (pH 7.8) following the addition of either 100 μmol/l NADH or 100 μmol/l NADPH; chemiluminescence was subsequently measured for 30 s by using a sensitive luminometer (TD-20/20; Turner Designs Inc., Sunnyvale, CA). Data are expressed as relative light units/s and are normalized against the total protein content.

Histopathology and immunohistochemistry

To assess the degree of fibrosis, the paraformaldehyde-fixed kidney tissues were embedded in paraffin and the 4 μm-thick sections were stained with Sirius red F3BA by using a previously described method [19]. The expression of the ED1 antibody (Chemicon International Inc., Temecula, CA) was determined using the avidin-biotin complex method (LSAB 2 kit; Dako Corp., Carpinteria, CA) and deparaffinized sections according to a previous method [12]. ED1 is a mouse monoclonal antibody to rat macrophages and is used to label blood monocytes and exudate macrophages [20]. In brief, the deparaffinized sections were pre-treated with 0.1% trypsin solution in phosphate-buffered saline (PBS; pH 7.4) and with 3% H_2O_2. The sections were then pre-incubated with 1.5% skim milk in PBS and incubated with the ED1 antibody (1:200). Thereafter, the sections were incubated by using the avidin-biotin complex method according to the manufacturer's protocol. Final incubation was carried out with streptavidin conjugated to horseradish peroxidase and positive reactions were visualized with 3,3′-diaminobenzidine tetrahydrochloride. The sections were counterstained with haematoxylin. For all the animals, cells showing a distinct immunoreaction for ED1 were counted in 50 randomly selected glomeruli and five randomly selected areas (0.0625 mm^2 each) in the corticomedullary junctions of the deparaffinized sections.

Computer-aided morphometrical analysis

To evaluate fibrosis in the glomeruli, the area stained red by Sirius red was measured using a colour image analyser (Mac Scope, Mitani Inc., Fukui, Japan) in five randomly selected glomeruli of the sections from all the animals. The percentage of the fibrotic area per unit of glomerulus was calculated.

Analysis for MCP-1 mRNA

Total RNA was isolated from each kidney by using a spin-vacuum (SV) total RNA isolation system (Promega, Madison, WI) according to the manufacturer's protocol. Transcripts from 1 μg of total RNA were reverse-transcribed with Pre-developed TaqMan assay reagents (Applied Biosystems, Foster City, CA). The resultant complementary DNA was amplified by real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR). PCR was monitored with a TaqMan ABI Prism 7000 Sequence Detection System. TaqMan gene-expression assays (primer/probe sets) specific for rat MCP-1 (assay ID, Mm00441242_m1) were purchased from Applied Biosystems. To control the amount of DNA available for RT-PCR in the different samples, gene expression of the target sequences was normalized in relation to the expression of an endogenous control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA (TaqMan Rodent GAPDH Control Reagent kit, Applied Biosystems).

Statistical analysis

Each value is expressed as mean ± SEM. Statistical analyses were performed using one-way analysis of variance (ANOVA), followed by the Tukey test.

Results

Effects of LP treatment on body and tissue weights and plasma parameters

No difference was observed in body weight gain between the body weights of the LP-treated and untreated diabetic rats (Table I). The relative weights of the kidneys of the diabetic rats were significantly higher than those

Table I. Morphological characteristics and plasma parameters.

	Control	STZ + LP treatments			
		0%	0.11%	0.33%	1.0%
BW (g)					
0 week	251 ± 5[a]	227 ± 5[b]	225 ± 2[b]	240 ± 4[a]	225 ± 6[b]
5 weeks	372 ± 8[a]	276 ± 12[b]	252 ± 9[b]	250 ± 10[b]	252 ± 13[b]
At sacrifice‡	366 ± 9[a]	253 ± 18[b]	243 ± 10[b]	231 ± 8[b]	231 ± 11[b]
Liver (g)	11.41 ± 0.31[a]	12.03 ± 0.71[a]	11.41 ± 0.57[a]	9.77 ± 0.28[b]	10.94 ± 0.38[a]
Kidney (g)	2.43 ± 0.06[a]	2.99 ± 0.10[b]	2.90 ± 0.11[b]	2.55 ± 0.07[a]	2.54 ± 0.08[a]
Heart (g)	0.941 ± 0.017[a]	0.813 ± 0.037[b]	0.743 ± 0.034[b]	0.705 ± 0.022[b]	0.741 ± 0.022[b]
LV (g)	0.176 ± 0.007	0.166 ± 0.008	0.146 ± 0.009	0.145 ± 0.008	0.150 ± 0.008
L/BW (g/kg)	31.19 ± 0.29[a]	47.81 ± 1.27[b]	46.91 ± 0.61[b]	42.46 ± 0.77[b,c]	47.69 ± 1.16[b]
K/BW (g/kg)	6.66 ± 0.15[a]	11.68 ± 0.68[b]	11.96 ± 0.21[b]	11.09 ± 0.25[b]	11.07 ± 0.33[b]
H/BW (g/kg)	2.58 ± 0.03[a]	3.25 ± 0.12[b]	3.06 ± 0.03[b]	3.06 ± 0.04[b]	3.23 ± 0.07[b]
LV/BW (g/kg)	0.483 ± 0.017[a]	0.664 ± 0.033[b]	0.597 ± 0.021[a]	0.631 ± 0.032[b]	0.654 ± 0.032[b]
pGlc (mg/dl)	167 ± 8[a]	504 ± 65[b]	846 ± 97[b,c]	464 ± 93[b]	432 ± 72[b]
BUN (mg/dl)	18.3 ± 0.8[a]	37.5 ± 3.5[b]	29.2 ± 2.0[b]	36.4 ± 1.6[b]	37.0 ± 2.1[b]
pCr (mg/dl)	0.091 ± 0.003[a]	0.173 ± 0.017[b]	0.172 ± 0.010[b]	0.146 ± 0.012[b]	0.129 ± 0.008[a]

‡At sacrifice after fasting overnight. BW, body weight; L, liver; K, kidney; H, heart; LV, left ventricule; pGlc, plasma glucose; BUN, blood urea nitrogen; and pCr, plasma creatinine. Data are mean ± SEM (n = 7–8). Means in a row without a common letter differ, $p < 0.05$.

of the control rats. Although there was no significant difference between the untreated and LP-treated diabetic rats, the latter showed a tendency for decreased relative weights of the kidneys. The plasma Glc levels of the diabetic rats were significantly higher than those of the control rats (Table I). Although the plasma Glc levels of the diabetic rats treated with 0.11% LP were significantly higher than those of the untreated diabetic rats, no difference was observed in the Glc levels between the untreated diabetic rats and those treated with 0.33% and 1.0% LP. This indicated that STZ caused irreversible damage to the pancreatic islet cells. The BUN and plasma Cr levels in the untreated diabetic rats were significantly higher than those in the control rats. Although there was no significant difference in the Cr levels between the untreated and LP-treated diabetic rats, the rats treated with 1.0% LP showed a tendency for decreased Cr levels.

Effects of LP treatments on the 8-OHdG levels in the 24 h urine samples

The 8-OHdG levels in the 24 h urine samples from the untreated diabetic rats were significantly higher than those in the samples from the control rats (Figure 2). Conversely, these levels were significantly lower in the LP-treated diabetic rats than in the untreated diabetic rats, indicating that treatment with LPs suppresses oxidative stress in STZ-induced diabetes.

Effects of LP treatments on superoxide production in the cortex and medulla of the kidney

Both NADH and NADPH stimulated O_2^- production in the cortices and medullae of the kidneys extracted from the STZ-induced diabetic rats. The NADPH-stimulated O_2^- levels in the renal cortices of the untreated diabetic rats were significantly higher than those in vehicle-injected control rats (Figure 3). Conversely, the O_2^- levels in the diabetic rats treated with 1.0% LP were significantly lower compared with those in the untreated diabetic rats. Similarly, the NADPH-stimulated O_2^- levels in the medullae of the diabetic rats fed an LP-free diet were significantly higher than those in the vehicle-injected control rats (Figure 3). Although no significant difference was detected between the untreated and LP-treated diabetic rats, the latter showed a tendency for reduction of NADPH-stimulated O_2^- levels.

Histopathological changes and ED1-positive macrophages in the renal cortices of STZ-induced diabetic rats

Representative Sirius red-stained renal cortical sections from the rats in the vehicle-injected controls, rats not treated with LP and those treated with 1.0%

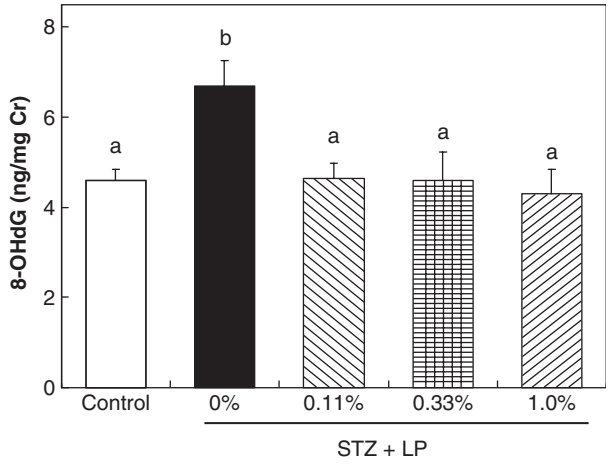

Figure 2. Effects of LP treatment on the 8-hydroxydeoxyguanosine levels in 24 h urine samples. values are expressed as mean ± SEM (n=6–7). Means without a common letter differ, p <0.05

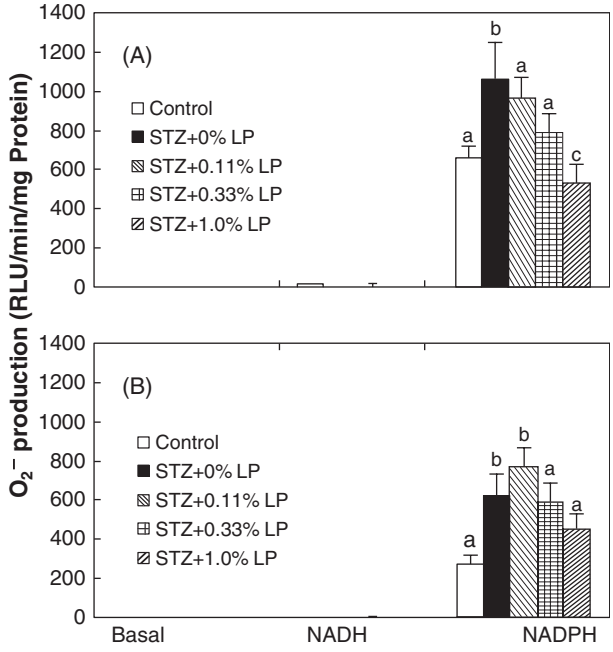

Figure 3. Effects of LP treatment on superoxide (O_2^-) production in the renal cortex (A) and medulla (B) of the control and STZ-induced diabetic rats under the basal (0 μmol/l) and NADH (100 μmol/l) and NADPH (100 μmol/l)-stimulated conditions. Data are expressed as mean±SEM (n=6–8). Means without a common letter differ, $p<0.05$.

LP are shown in Figure 4. The basement membrane and mesangial matrix in the glomeruli of the untreated diabetic rats appeared expanded compared with those of the control rats. In comparison, the expansion of the basement membrane and mesangial matrix was less pronounced in the diabetic rats treated with 1.0% LP. To estimate the effects of the LP treatments on glomerular fibrosis, the glomerular area stained with Sirius red was measured using an image analyser. The percentage of the fibrous area per unit glomerular volume was significantly greater in the untreated diabetic rats than in the control rats (Figure 5). In the diabetic rats treated with 0.33% and 1.0% LP, the percentage of fibrotic areas was significantly lower than that in the untreated diabetic rats. These results indicated that the LP treatments attenuated the degree of glomerular fibrosis in the diabetic rats.

Effects of LP treatments on the kinetics of ED1-positive macrophages in the kidneys of STZ-induced diabetic rats

As shown in Figure 4, immunohistochemical analyses revealed ED1-positive macrophages in the glomeruli of both LP-treated and untreated diabetic rats. The number of ED1-positive macrophages in the untreated diabetic rats was significantly greater than that in the vehicle-injected control rats. In contrast, the number of macrophages in the diabetic rats treated with 1.0% LP was significantly lower than that in the untreated diabetic rats. To examine the effects of LP treatments on the presence of macrophages in the diabetic rat kidney, we counted the number of ED1-positive macrophages in the glomeruli and tubulointerstitium of the corticomedullary junction (Figure 6). The number of ED1-positive macrophages in the untreated diabetic rats was significantly higher than that in the control rats. In contrast, the number of macrophages in the LP-treated diabetic rats was significantly lower than that in the untreated diabetic rats. These data indicate that the LP treatments attenuated the infiltration of macrophages into both the glomerular and tubulointerstitial areas in the diabetic rats.

Effect of LP treatments on the expression of MCP-1 mRNA in the renal cortices of STZ-induced diabetic rats

Although there was no significant difference between the MCP-1 mRNA expression levels in the renal cortices of the vehicle-injected control rats and the untreated diabetic rats, the latter showed a tendency for increased MCP-1 mRNA levels (Figure 7). MCP-1 mRNA expression in the diabetic rats treated with 1.0% LP was significantly lower than that in the untreated diabetic rats, indicating that LP treatment suppresses MCP-1 mRNA in the kidneys of the diabetic rats.

Discussion

LPs are derivatives of lignin, which is a component of plant cell walls. LPs are known to be highly phenolic and to exhibit antioxidant properties *in vitro* [13,14]. However, the physiological roles underlying the beneficial effects of LPs on diabetic nephropathy have not yet been clarified.

The major findings of the present study are as follows: (i) LP treatment attenuated oxidative stress in STZ-induced diabetic rats as indicated by the levels of 8-OHdG in the 24 h urine samples and the NAD(P)H oxidase-stimulated O_2^- production in the renal cortex and medulla; (ii) LP treatment suppressed the increase in the number of infiltrating macrophages in the glomerular and tubulointerstitial areas as compared to the number in the untreated diabetic rats; and (iii) LP treatment suppressed the expression of MCP-1 mRNA, which is involved in the infiltration and activation of macrophages in the renal cortices of the diabetic rats.

There is emerging evidence that the formation of ROSs is a direct consequence of hyperglycaemia. Increased levels of 8-OHdG, which is a marker of oxidative DNA damage, have been reported in the kidneys of diabetic rats and in the tissues and body fluids of diabetic patients [21,22]. We also demonstrated that the urinary 8-OHdG levels were significantly higher in the untreated diabetic rats than in the vehicle-injected control rats. Further, the 8-OHdG levels in the LP-treated rats were significantly lower than those in the untreated rats, suggesting that LP treatment suppresses the increase in oxidative stress in STZ-induced diabetic rats.

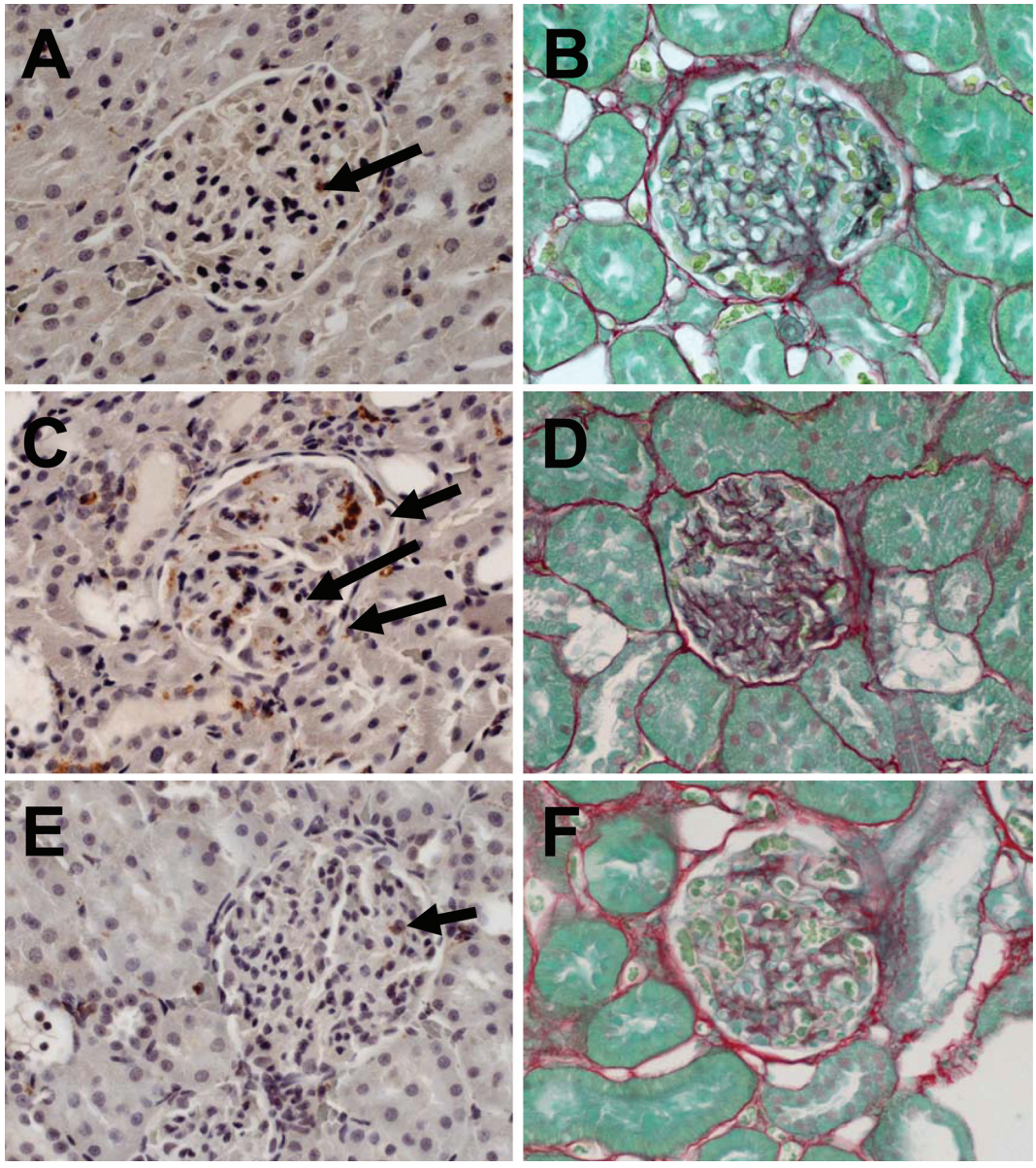

Figure 4. Representative images of Sirius red-stained sections and immunohistochemical staining of macrophage-specific EDI in the rat glomeruli. (A, B): control rats, (C, D): untreated diabetic rats and (E, F): diabetic rats treated with 1.0% LP. Sirius red staining in (A, C, E). EDI-positive macrophages are shown with arrows in (B, D, F). Counterstaining with haematoxylin has been carried out. Original magnification, ×600.

The pathogenesis of diabetic nephropathy also involves oxidative stress [23,24]. In this study, we demonstrated that the O_2^- production in the renal cortex and medulla was significantly higher in the untreated diabetic rats than in the control rats. In contrast, the O_2^- production in the renal cortices of the LP-treated rats was significantly lower than that in the renal cortices of the untreated rats (Figure 3). These results suggest that the LP treatments attenuated diabetic nephropathy via the reduction of O_2^- in the renal cortices of STZ-induced diabetic rats.

Our histological analyses revealed that Sirius red staining in the glomeruli was markedly weaker in the LP-treated diabetic rats than in the untreated diabetic rats (Figure 4). Glomerular expansion has been reported to be a characteristic feature of experimental diabetic animals and of humans with diabetes mellitus [7,25,26]. Morphometrical analysis revealed that the areas of glomerular fibrosis in the LP-treated diabetic rats were significantly smaller than those in the untreated diabetic rats (Figure 5). In addition, the kidney weight returned to normal in the diabetic rats treated with 1.0% LP but not in the control rats and the plasma Cr levels in the LP-treated diabetic rats showed a tendency to decrease (Table I). These results reflected that treatment with LPs may attenuate diabetic nephropathy in the STZ-induced rat model.

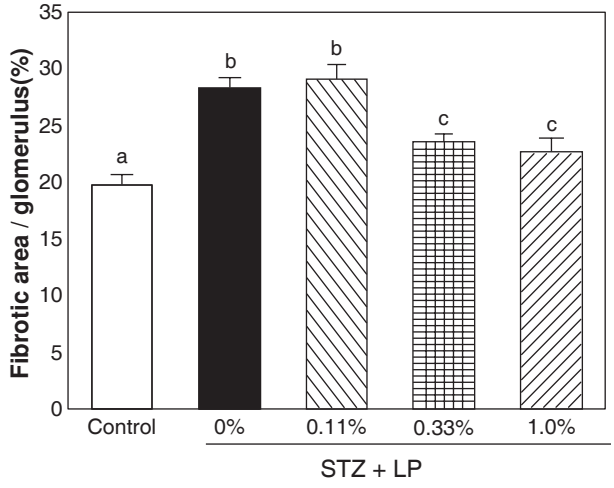

Figure 5. Effects of LP treatment on the area of fibrosis per unit glomerular volume in the control and STZ-induced diabetic rats. Values are expressed as mean±SEM (n=7–8). Means without a common letter differ, p <0.05.

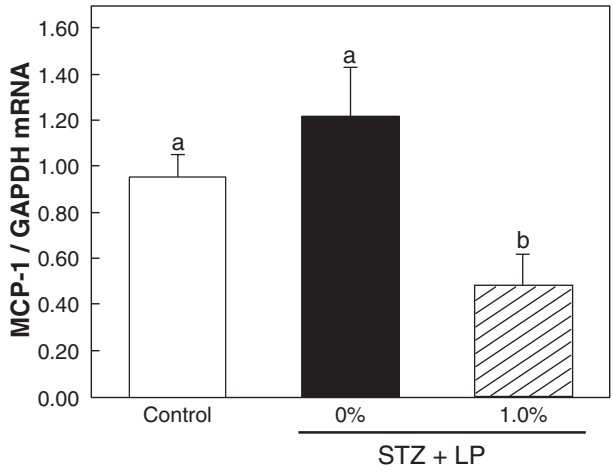

Figure 7. Expression of MCP-1 mRNA in the kidneys of the vehicle-injected control rats, untreated diabetic rats and diabetic rats treated with 1.0% LP. Values are indicated as arbitrary ratios of the amount of expressed mRNA of each factor to that of GADPH. Values are expressed as mean±SEM (n=5–7). Means without a common letter differ, p <0.05.

More interestingly, LPs, which are highly phenolic, significantly reduced the number of ED1-positive macrophages in the glomerular and tubulointerstitial areas in the LP-treated diabetic rats relative to the number observed in the untreated diabetic rats (Figure 4). Previous studies have reported an increased number of glomerular macrophages in diabetic animals [27–29]. These infiltrating macrophages have been considered to contribute to the progression toward glomerulosclerosis by producing fibrogenic factors such as transforming growth factor-β and platelet-derived growth factor [30,31]. From our findings in this study, LPs may be expected to suppress the infiltration of macrophages in the glomerular and tubulointerstitial areas in rats with diabetic nephropathy.

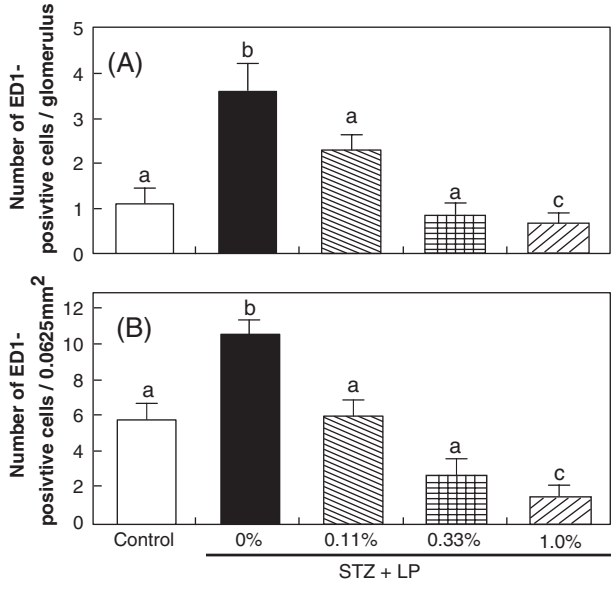

Figure 6. Effects of LP treatment on the number of EDI-positive macrophages in the glomeruli (A) and in the tubulointerstitium of the corticomedullary junctions (B) in the control and STZ-induced diabetic rats. Values are expressed as mean±SEM (n=7–8). Means without a common letter differ, p <0.05.

The reasons for the attenuation of STZ-induced diabetic nephropathy by LP treatment remain unclear. One possibility is that LPs, which are highly phenolic [13], may be associated with the expression of MCP-1 mRNA in the kidneys of STZ-induced diabetic rats. In our study, the RT-PCR analyses revealed that renal MCP-1 mRNA expression was significantly lower in the LP-treated diabetic rats than in the untreated diabetic rats (Figure 7). Several studies have showed that MCP-1 plays an important role in the infiltration and activation of macrophages in diabetic nephropathy [32,33]. In addition, macrophages seem likely to promote fibroblast proliferation via the Interleukin-1 (IL-1) and platelet-derived growth factor (PDGF)-dependent pathway, which is associated with progressive fibrosis in diabetic nephropathy [7,34]. Therefore, we hypothesized that LP treatment may, at least in part, reduce macrophage infiltration by suppressing the upregulation of MCP-1 expression in the renal cortex in STZ-induced diabetic rats. Another possible reason for the attenuation of STZ-induced diabetic nephropathy by LPs is that LPs may act as inhibitors of NAD(P)H oxidase and/or suppressors of NAD(P)H oxidase sub-units in the renal cortex and medulla. This supposition is based on the finding that LP treatment attenuated the production of NAD(P)H oxidase-derived ROSs, particularly O_2^-, in the renal cortex of STZ-induced diabetic rats. The pathogenesis of diabetic nephropathy involves oxidative stress and the activation of NAD(P)H oxidase in the kidney [23,35]. Several studies have reported that the activity of NAD(P)H oxidase in the glomeruli of diabetic rats is much higher than that in the glomeruli of control rats

[23,36] and that the renal expression of NAD(P)H oxidase sub-units is enhanced in diabetic rats [37,38]. Apocynin (4-hydroxy-3-methoxyacetophenone), a methoxy-substituted catechol, is known to inhibit NAD(P)H oxidase by impeding the assembly of the p47phox and p67phox sub-units [39]. Since the chemical structure of apocynin is very similar to that of LPs, we assumed that LPs may reduce NAD(P)H oxidase-derived O_2^- production in the renal cortex by suppressing the activation and/or expression levels of NAD(P)H oxidase sub-units in rats with diabetic nephropathy.

In conclusion, we herein demonstrated that LP treatment suppresses excess oxidative stress and increases the number of infiltrating macrophages, expression of MCP-1 mRNA and glomerular expansion in STZ-induced diabetic kidneys. Although the mechanisms underlying the effects of LPs on oxidative stress and macrophage infiltration in diabetic nephropathy need to be investigated further, the current results may facilitate the formulation of preventive-care strategies for diabetic nephropathy.

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