Free Radical Research, May 2011; 45(5): 575–584

informa
healthcare

RESEARCH ARTICLE

Telmisartan attenuates oxidative stress and renal fibrosis in streptozotocin induced diabetic mice with the alteration of angiotensin-(1–7) *mas* receptor expression associated with its PPAR-γ agonist action

ARUN PRASATH LAKSHMANAN[1], KENICHI WATANABE[1], RAJARAJAN A. THANDAVARAYAN[1,2], FLORI R. SARI[1], MEILEI HARIMA[1], VIJAYASREE V. GIRIDHARAN[2], VIVIAN SOETIKNO[1], MAKOTO KODAMA[3] & YOSHIFUSA AIZAWA[3]

[1]*Department of Clinical Pharmacology, Faculty of Pharmaceutical Sciences, Niigata University of Pharmacy and Applied Life Sciences, Niigata City, Japan,* [2]*Department of Functional and Analytical Food Sciences, Niigata University of Pharmacy and Applied Life Sciences, Niigata City, Japan, and* [3]*First Department of Internal Medicine, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan*

(Received date: 24 November 2010; Accepted date: 25 January 2011)

Abstract

The beneficial effects of telmisartan on Angiotensin (Ang)-II mediated oxidative stress and renal fibrosis in streptozotocin (STZ)-induced diabetic nephropathy (DN) were studied. Thirty mice were divided into normal (NG), STZ-induced diabetic (DG) and telmisartan-treated diabetic (TG) groups. Compared with NG mice, DG mice showed significant up-regulations of AT-1R, TGF-β1, p-p38MAPK, p-MAPKAPK-2, p-Akt, p47phox, p67phox, gp91phox protein and collagen-III and all of these were significantly reversed in TG mice. The down-regulated protein expression of Ang-(1–7) *mas* receptor, ACE-2, PPAR-γ and PGC-1α were observed in DG mice and a significant up-regulation effect of telmisartan has been seen in the TG mice. Furthermore, TG mice showed reduced expression of fibronectin, production of superoxide radical as well as renal hypertrophy and fibrosis when compared with DG mice. These findings suggest that Ang-II plays a significant role in DN and telmisartan would be beneficial in reducing oxidative stress and fibrosis in STZ-induced DN.

Keywords: *Renin-angiotensin-aldosterone system, diabetic nephropathy, oxidative stress, fibrosis, telmisartan*

Introduction

Diabetic nephropathy (DN) is a common complication of diabetes mellitus (DM) and represents one of the major challenges for modern nephrology as the most common cause of end-stage renal disease (ESRD), accounting for ~ 40% of new cases [1,2]. The mortality of DN patients is high and a marked increase in cardiovascular risk accounts for more than one-half of the excess deaths seen in these patients. Once overt DN is present, ESRD can be postponed, but usually not prevented, even by effective anti-hypertensive treatment and careful glycemic control [3,4].

DN is characterized by hypertrophy of the glomerular and tubular structures of the kidneys, thickening of the basement membrane, glomerular hyperfiltration and accumulation of extracellular matrix components in the glomerular mesangium and tubular interstitium [5,6]. Several researchers have implicated the involvement of the renin-angiotensin system (RAS), oxidative stress and fibrosis during the DN. The RAS is known to play a central role in the control of blood pressure, fluid volume and sodium balance and the overactivity of this system contributes to the pathogenesis of various clinical conditions, including mainly DN.

Correspondence: Kenichi Watanabe, Department of Clinical Pharmacology, Faculty of Pharmaceutical Sciences, Niigata University of Pharmacy and Applied Life Sciences, 265-1 Higashijima Akiha Ku, Niigata City 956-8603, Japan. Tel: +81 250 25 5267. Fax: +81 250 25 5021. Email: watanabe@nupals.ac.jp

ISSN 1071-5762 print/ISSN 1029-2470 online
DOI: 10.3109/10715762.2011.560149

Angiotensin (Ang)-II is the most important active factor in the RAS and it exerts many haemodynamic as well as non-haemodynamic effects on renal cells that may contribute to the progression of the DN [7]. It produces nephrotoxic reactive oxygen species (ROS) and stimulates cell proliferation and tissue remodelling by enhancing the synthesis of profibrotic cytokines such as transforming growth factor (TGF)-β1 and other growth factors. Collagen–III deposition is also enhanced through the inhibition of proteases that normally function to degrade abnormal tissue proteins [8,9]. The ROS also play an important role in oxidative stress and, thus, modifying the extracellular matrix proteins by converting nitric oxide (NO) to cytotoxic peroxynitrite is the key point of extracellular matrix (ECM) degradation by the ROS [10,11]. Increased ROS during DM further activate various pathways including advanced glycation end-products (AGEs) and protein kinase C-dependent activation of NAPDH oxidase [12,13]. Taken together, based on the homeostatic effects of RAS on the kidney, it is evident that a proper function of this cascade is required to maintain the normal renal function. Anti-hypertensive medications that modulate the RAS do so by angiotensin converting enzyme (ACE), angiotensin receptor blockade (ARB) or aldosterone receptor antagonism. The angiotensin receptor blockers (ARBs) are showing similar effects like ACE inhibitor, in controlling the blood pressure and in treating the DN [14]. Recently, randomized clinical trials have demonstrated that the angiotensin-II type 1 receptor (AT-1R) antagonist, losartan, has significant renoprotective effects on non-diabetic [15] as well as DN. Sugiyama et al. [16] have reported that another AT-1R antagonist, telmisartan, inhibited oxidant-mediated tissue injury and NADPH oxidase sub-units (p22phox, p47phox and p67phox). Additionally, Ang-II has been reported to play critical roles in the pathogenesis of renal interstitial fibrosis [17]. Satoh et al. [18] have reported that renal fibrosis was significantly reduced in the AT-1Ra gene knockout mice after unilateral ureteral obstruction (UUO). Seeland et al. [19] have reported that telmisartan reduces myocardial hypertrophy and interstitial fibrosis in TGF-β1 transgenic mice. Furthermore, Yao et al. [20] have reported that telmisartan inhibited TGF-β1-induced alpha-smooth muscle actin expression and collagen IV secretion in mesangial cells via the activation of peroxisome proliferator activating receptor-γ (PPAR-γ) [20].

Despite the significant roles of Ang-II in the pathogenesis of DN, the roles of AT-1R antagonist in Ang-II mediated renal tissue injury are largely unknown. Therefore, we investigated whether the AT-1R antagonist, telmisartan, could inhibit the Ang-II mediated oxidative stress, renal tissue injury and renal fibrosis in STZ-induced DN mice.

Materials and methods

Materials

Unless otherwise stated all reagents were of analytical grade and purchased from Sigma (Tokyo, Japan). Telmisartan was generously provided by Boehringer Ingelheim GmbH (Ingelheim am Rhein, Germany).

Diabetes induction

Diabetes was induced by a single intraperitoneal (i.p.) injection of STZ (Sigma, St Louis, MO) at a dose of 150 mg/kg body weight (BW) to 8–10-weeks-old male C57BL/6 JAX mice which was obtained from Charles River Japan Inc. (Kanagawa, Japan). STZ was dissolved in 20 mM sodium citrate saline buffer (pH 4.5) and injected within 5 min of preparation. Age matched male C57BL/6 JAX mice were injected with 100 µl of citrate buffer and they were used as non-diabetic normal mice. Mice were maintained with free access to water and chow throughout the period of study and they were treated in accordance with the guidelines from animal experimentation of our institute.

Experimental protocol

After 3 days of STZ injection, the blood glucose (BG) levels were measured using Medi-safe chips (Terumo Inc., Tokyo, Japan). The mice showing blood glucose level > 300 mg/dL were considered as diabetic and included in this study. Thirty mice were divided into the following three groups: (1) Vehicle-treated normal (non-STZ induced) group (NG; $n = 10$); (2) vehicle-treated diabetic group (DG; $n = 10$); (3) telmisartan-treated diabetic group (TG; $n = 10$). Telmisartan was dissolved into 0.5% hydroxy ethyl cellulose (HEC) and given orally for 28 days at a dose of 5 mg/kg BW. The NG and DG mice received 0.5% HEC alone. After 28 days of STZ injection or telmisartan treatment, mice were anaesthetized with a single i.p. injection of pentobarbital (50 mg/kg BW) and the kidneys were excised and decapsulated. The kidney weight (KW) and the ratio of KW to BW (KW/BW) were determined for each animal. Half of the kidney was immediately snap-frozen in liquid nitrogen and stored at –80°C for subsequent protein analysis. The remaining excised kidneys were fixed in 10% formalin and frozen for histopathological studies.

Western blotting

Protein lysate was prepared from the kidney as described previously [21]. The total protein concentration in samples was measured by the bicinchoninic acid (BCA) method [22]. For the determination of protein levels of AT-1R, angiotensin-II type 2 receptor (AT-2R), Ang-(1–7) *mas* receptor, angiotensin

converting enzyme (ACE)-2 and NADPH oxidase sub-units (p47phox, p67phox and gp91phox) and nephrin, peroxisome proliferator activating receptor-γ (PPAR-γ), transforming growth factor (TGF)-β1, phospho p38 MAPK (p-p38 MAPK), phospho mitogen activated protein kinase-activated protein kinase-2 (p-MAPKAPK-2), protein kinase B (p-Akt), collagen-III and PPAR-γ co-activator-1α (PGC-1α) equal amounts of protein extracts (30 μg) were separated by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (Bio-Rad, CA) and transferred electrophoretically to nitrocellulose membranes. Membranes were blocked with 5% non-fat dry milk in Tris buffered saline Tween (20 mM Tris, pH 7.6, 137 mM NaCl and 0.1% Tween 20). Primary antibodies against AT-1R, AT-2R, ACE-2 (PPAR-γ), p47phox, p67phox, gp91phox, nephrin, PGC-1α and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were obtain from Santa Cruz Biotechnology (Santa Cruz, CA). Primary antibodies against p-p38 MAPK, p-MAPKAPK-2 and p-Akt were obtained from Cell Signaling Technology Inc. (Beverly, MA).

Primary antibodies against Ang-(1–7) *mas* receptor were obtained from Alomone Labs Ltd. (Jerusalem, Israel). Primary antibody against TGF-β1 was obtained from Promega (Madison, WI). Primary antibody against collagen-III was obtained from Abcam (Cambridge, UK). All the antibodies were used at a dilution of 1:1000. The membrane was incubated overnight at 4°C with the primary antibody and the bound antibody was visualized using the respective horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology Inc.) and chemiluminescence developing agents (Amersham Biosciences, Buckinghamshire, UK). The level of GAPDH was estimated in every sample to check for equal loading of samples. Films were scanned and band densities were quantified with densitometric analysis using Scion Image program (Epson GT-X700, Tokyo, Japan). All values were normalized by setting the density of normal samples as 1.0.

Light microscopy morphological study

The kidney tissue was cut into 2-mm-thick transverse slices and fixed in 10% formalin. After being embedded in paraffin, several transverse sections were obtained from kidney and stained with HE. Also, the samples were stained with Azan-Mallory to demonstrate fibrosis in the kidney. The frequency and severity of kidney lesions were assessed semi-quantitatively, as previously reported [23,24].

Formalin-fixed, paraffin-embedded kidney tissue sections were used for immunohistochemical staining. After deparafinization and hydration, the slides were washed in Tris-buffered saline (TBS; 10 mmol/l Tris-HCl, 0.85% NaCl, pH 7.5) containing 0.1% bovine serum albumin (BSA). Endogenous peroxidase activity was quenched by incubating the slides in methanol and 0.6% H_2O_2/methanol. To perform antigen retrieval, the sections were pre-treated with trypsin for 15 min at 37°C. Blocking was done with normal goat serum for fibronectin. After overnight incubation with anti-fibronectin antibodies (1:50 dilution) at 4°C, the slides were washed in TBS buffer and HRP conjugated secondary antibody (Santa Cruz) was added and incubated at room temperature for 45 min. The immuno-staining was visualized using diamino benzidine tetrahydrochloride (DAB), the slides were counterstained with hematoxylin. Measurements of fibrosis and endothelial function were made by counting the average number of stained cells under 400-fold magnification using a light microscope. For all, 50 random fields representing the whole section were examined per section and three animals were used per group.

In situ detection of superoxide production in kidney

To evaluate *in situ* superoxide production from kidney, unfixed frozen cross-sections of the specimens were stained with dihydroethidium (DHE; Molecular Probes, Eugene, OR) according to the previously validated method [25]. In the presence of superoxide, DHE is converted to the fluorescent molecule ethidium, which can then label nuclei by intercalating with DNA. Briefly, the unfixed frozen tissues were cut into 10-μm-thick sections and incubated with 10 μM DHE at 37°C for 30 min in a light-protected humidified chamber. Fluorescence images were obtained using a fluorescence microscope equipped with a rhodamine filter.

Statistical analysis

Data are represented as means ± standard error of mean (SEM). Statistical analysis of differences between groups was performed by student's *t*-test using GraphPad Prism 5.0 software. Differences were considered significant at $p < 0.05$.

Results

Effect of telmisartan on BW, hyperglycemia and KW/BW ratio

A significant increase of BG level was observed in the DG mice at 3 days after i.p. injection of STZ when compared with NG mice. The BW of the DG mice was significantly reduced when compared with the NG mice. There were no significant changes in BW and BG level in the TG mice when compared with the DG mice. There was no change in KW/BW ratio among the groups (Table 1).

Table 1. Changes in body weight, blood glucose, kidney weight and KW/BW ratio after STZ treatment alone or in combination with telmisartan (5 mg/kg BW).

Group	Body weight (g)	Blood glucose concentration (mg/dL)	Kidney weight (g)	KW/BW ratio
NG	31.33 ± 0.59	163 ± 19.90	0.173 ± 0.004	0.005 ± 0.002
DG	25.60 ± 1.14**	472 ± 23.60**	0.199 ± 0.007	0.007 ± 0.003
TG	24.90 ± 0.88$$	484 ± 14.37$$	0.185 ± 0.010	0.007 ± 0.003

**p<0.01 vs NG, $$p<0.01 vs NG

Effect of telmisartan on the protein expression of AT-1R, AT-2R, Ang-(1–7) mas receptor and ACE-2 in DN

Induction of diabetes by STZ elevated the renal protein expression of AT-1R and AT-2R in the DG mice when compared with that of the NG mice. Renal protein expression of AT-1R was significantly reduced in the TG mice when compared with the DG mice. The levels of Ang-(1–7) *mas* receptor and ACE-2 were lowered in DG mice in comparison to NG mice and significantly elevated in the TG mice in comparison to the DG mice (Figure 1).

Effect of telmisartan on NADPH subunits in DN

In the DG mice, the renal protein expression of NADPH sub-units including p47phox, p67phox and gp91phox was increased when compared with the NG mice. In the TG mice we could see the significant reduction in the expression of NADPH oxidase sub-units when compared with the DG mice (Figure 2).

Effect of telmisartan on free radical production in STZ-induced diabetic kidney

In the DG mice, there was an increased production of free radical when compared with the NG mice. Treatment with telmisartan significantly attenuated the production of free radical in diabetic kidney (Figure 3A).

Effect of telmisartan on the expression of PPAR-γ, p-p38 MAPK, p-MAPKAPK-2, p-Akt and nephrin

In the DG mice we could see the increased phosphorylation of p38 MAPK, MAPKAPK-2 and Akt and decreased expression of PPAR-γ protein in comparison to the NG mice. Conversely, the TG mice showed reduced phosphorylation of renal p38 MAPK, MAPKAPK-2 and Akt and increased PPAR-γ protein expression. There was no significant change in the expression of nephrin among the groups (Figures 4A and 5).

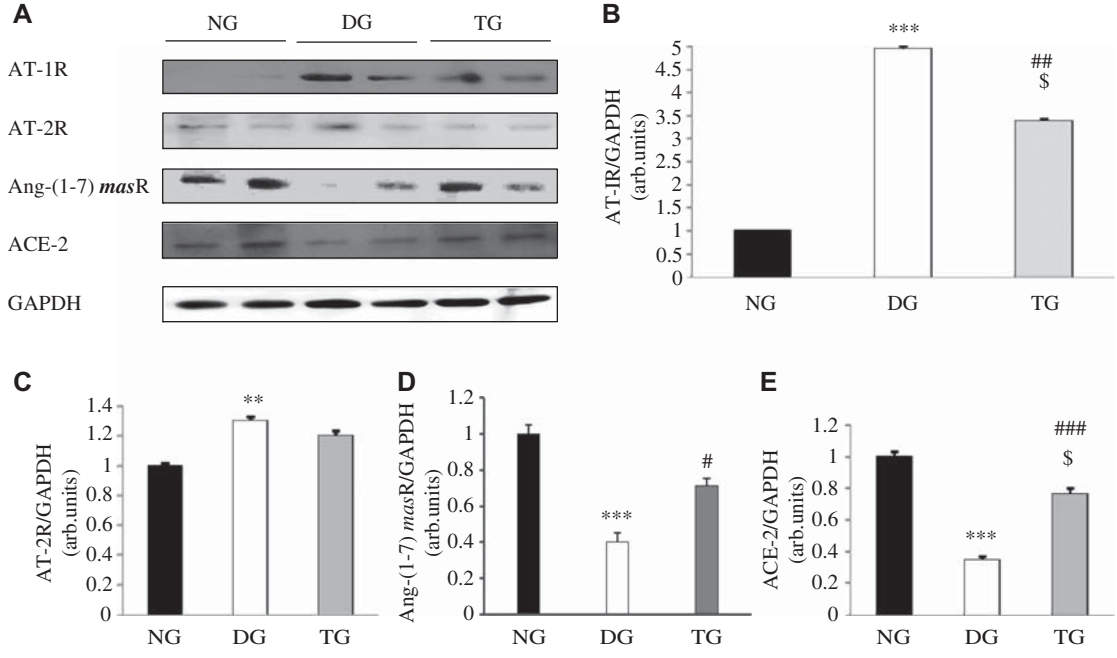

Figure 1. Effect of telmisartan on the renal expression of AT-1R, AT-2R, Ang-(1–7) *mas* receptor and ACE-2 in the DG mice. Representative Western blots (A) showing specific bands for AT-1R, AT-2R, Ang-(1–7) *mas* receptor, ACE-2 and GAPDH as an internal control and quantified by using densitometric analysis (B–E). An equal amount of protein sample obtained from kidney homogenate was applied to each lane. In the DG mice, telmisartan treatment significantly reduced the protein expression of AT-1R and no change in the expression of AT-2R. In DG mice, telmisartan treatment significantly up-regulated the protein expression of Ang-(1–7) *mas*R and ACE-2. NG, vehicle only treated group; DG, STZ induced diabetic group; TG, telmisartan (5 mg/kg BW) treated diabetic group. All values are expressed as the mean ± SEM; $n = 5$; $^{**}p < 0.01$ and $^{***}p < 0.001$ vs NG, $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ vs DG, $^{\$}p < 0.05$ vs NG.

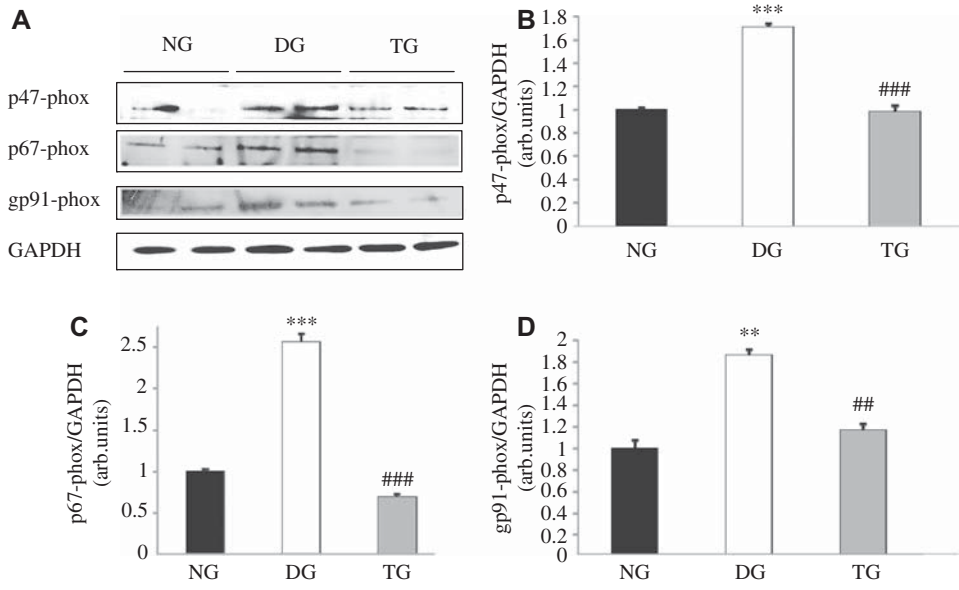

Figure 2. Effect of telmisartan on the renal expression of NADPH sub-units like p47-phox, p67-phox and gp91-phox in DN mice. Representative Western blots (A) showing specific bands for p47-phox, p67-phox and gp91-phox and GAPDH as an internal control and quantified by using densitometric analysis (B–D). An equal amount of protein sample obtained from kidney homogenate was applied to each lane. In DN mice, telmisartan treatment significantly reduced the protein expression of p47-phox, p67-phox and gp91-phox. NG, vehicle only treated group; DG, STZ induced diabetic group; TG, telmisartan (5 mg/kg BW) treated diabetic group. All values are expressed as the mean ± SEM; $n = 5$; $^{**}p < 0.01$ and $^{***}p < 0.001$ vs NG, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ vs DG.

Effect of telmisartan on hypertrophy and fibrosis in DN

The western blot analysis has also shown the increased expression of TGF-β1 and collagen-III in the DG mice than in the NG mice (Figures 4B and 6C). HE and Azan Mallory staining showed increased renal hypertrophy and fibrosis ($^{**}p < 0.01$ vs NG; $\#p < 0.05$ vs DG). There was also an increased expression of fibronectin in the DG mice when compared to the NG mice (Figure 3B). In addition, the TG mice showed significant reduction in the renal hypertrophy (DG, 46.26 ± 4.6 vs TG, 27.65 ± 2.9) and renal fibrosis (DG, 4.04 ± 0.21 vs TG, 2.21 ± 0.01) when compared with the DG mice (Figures 3C and D).

Discussion

The RAS is playing an important role in the pathophysiology of DM-related complications, mainly in the DN. Maladaptive up-regulation of RAS increases the production of Ang-II and aldosterone, which further leads to the progression of kidney damage through the excessive production of nephrotoxic ROS and the stimulation of cell proliferation [10,11]. The INNOVATION and AMADEO studies have explained the beneficial role of the AT-1R antagonist in the management of DN, which is widely proved to act through the RAS [26]. Nakao et al. [15] have reported the positive role of AT-1R antagonists in the protection of renal damage. However, still the mechanism whereby the reduction of nephrotoxic ROS and fibrosis by AT-1R antagonist in STZ-induced DN is unclear. We investigated the beneficial effects of telmisartan on Ang-II mediated oxidative stress and fibrosis in mice with DN induced by i.p. injection of STZ.

Recent studies have clearly explained the involvement of ang-II in kidney disease for the activation of oxidative stress, fibrosis and inflammatory process. Several receptors and enzymes of RAS, such as AT-1R, AT-2R, Ang-(1–7) *mas* receptor and ACE-2, are playing an important role in the modulation of RAS function. In STZ-induced DN, there would be an increased protein expression of AT-1R and AT-2R. The enzyme chemically related to ACE, known as ACE-2, showed significant involvement in modulating RAS by regulating number of angiotensins, including angiotensin (Ang)-I, Ang-II, Ang-(1–7) *mas* receptor and Ang-(1–9). ACE-2 produces its vasodilatory action mainly by converting Ang-II to Ang-(1–7) [27], probably through the *mas* receptor [28], and the ratio of Ang-(1–7) to Ang-II formed from Ang-I was lower in glomeruli of diabetic rats [29]. Hyperglycemia caused by STZ leads to decreased protein expression of ACE-2 through the activation of Ang-II and increased pro-oxidant effects in kidney, which might ultimately reduce the protein expression of Ang-(1–7) *mas* receptor. This is well supported by the findings of Tikellis et al. [30], who reported that the renal ACE-2 is reduced in the proximal tubules of the STZ-induced diabetes and the attenuation of renal injury by ACE inhibition is associated with increased ACE-2 expression. All these Ang-II mediated effects were expected to reverse by treatment with telmisartan. We also

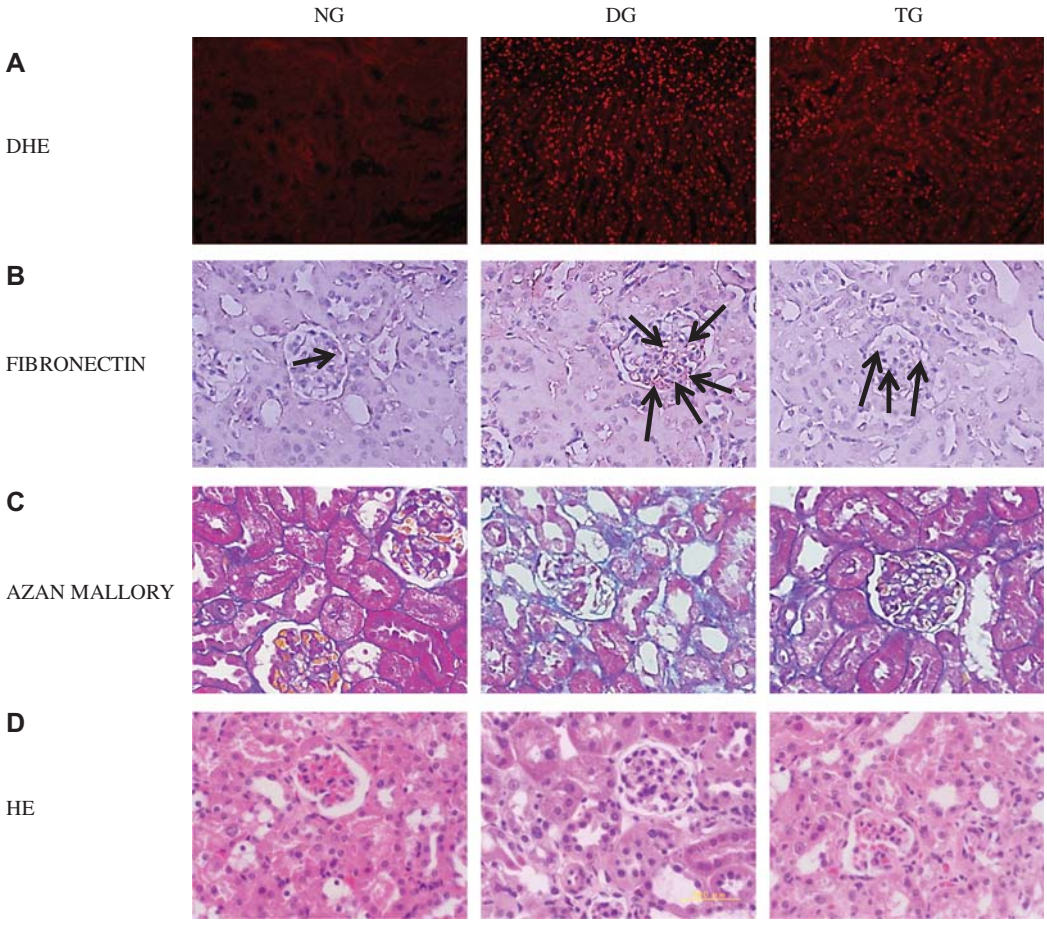

Figure 3. (A and B) DHE staining and immunohistochemistry for fibronectin in normal, STZ-induced diabetic and telmisartan-treated diabetic mice, respectively. In DN mice, telmisartan treatment effectively reduced expression of free radical production and fibronectin. NG, vehicle only treated group; DG, STZ-induced diabetic group; TG, telmisartan (5 mg/kg BW) treated diabetic group. Representative photomicrographs of neutral formaldehyde (10%)-fixed and unfixed frozen sections from kidney. Magnification × 400. (C and D) Azan-Mallory staining for fibrosis and HE staining for renal hypertrophy of cross-sectional kidney tissue slices (A) NG, vehicle only treated group; (B) DG, STZ-induced diabetic group; (C) TG, telmisartan (5 mg/kg BW) treated group; TG mice showed significant reduction in the area of fibrosis in comparison to the DG mice. Furthermore, TG mice showed reduced renal hypertrophy when compared with DG mice. Magnification × 400 and quantitative analysis of fibrosis in kidney. Fibrosis is indicated by the blue area as opposed to the red area.

found that the DG mice showed increased renal expression of AT-1R and decreased expression of ACE-2 and Ang-(1–7) *mas* receptor when compared with the NG mice. The TG mice in comparison to the DG mice showed significant reduction of renal protein expression of AT-1R and significant up-regulation of ACE-2 and Ang-(1–7) *mas* receptor (Figure 1), which suggest that the telmisartan treatment might increase the production of Ang-(1–7) *mas* receptor from Ang-II through the up-regulation of ACE-2. This increased formation of Ang-(1–7) *mas* receptor would have beneficial effects in STZ-induced renal dysfunction in mice.

The induction of hyperglycemia by STZ activates the renal RAS which leads to oxidative damage through the production of ROS by NADPH oxidase [31]. It has been reported that the enhanced NADPH oxidase activity is associated with oxidative damage to DNA in diabetic glomeruli [32,33]. Several different studies have demonstrated the role for ROS in the development of different diseases, especially in kidney and heart, in which Ang-II is a central component. Since the formation of ROS is dependent on multi-level pathways, recent studies indicate that NADPH oxidase is a major source of ROS in renal cells such as tubular epithelial cells and glomerular mesangial cells (MCs) [34–36]. The cytosolic p47phox plays an important role in Ang-II and tumour necrosis factor-α (TNF-α) induced NADPH oxidase activation. The post-phosphorylation of cytosolic p47phox will generate various free radicals like superoxide, hydrogen peroxide and hydroxyl radicals [18,37], which are leads to renal tissue injury. Onozato et al. [38] have reported that blockade of AT-1R causes decreased expression of p47phox and production of O_2^- in the kidney. Several reports stated that gp91phox were also up-regulated in the diabetic rats [39]. Consistent with previous results, in the present study, the renal expression of p47phox, p67phox and gp91phox were significantly increased in the DG mice in comparison to NG mice and the expression of those proteins were significantly reduced in the TG mice when compared

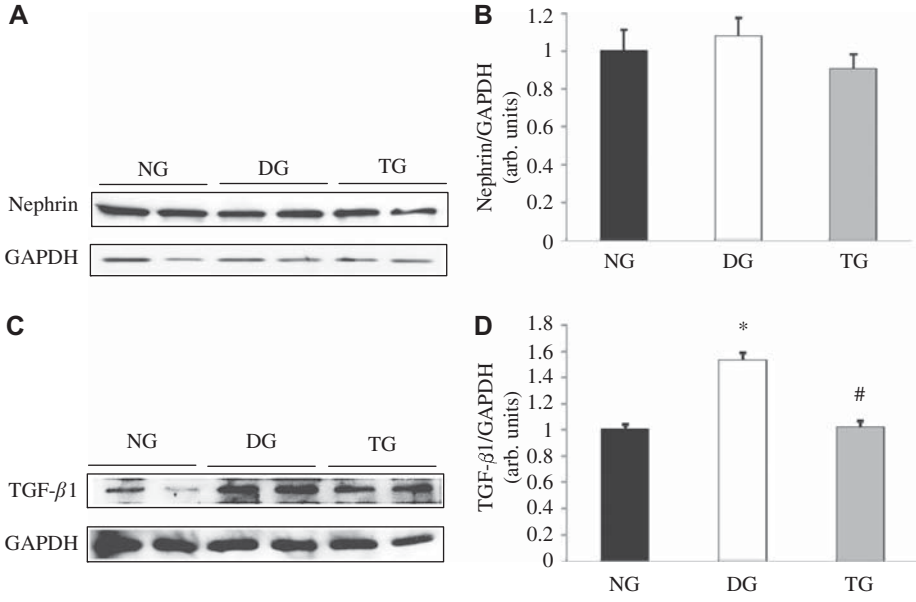

Figure 4. Effect of telmisartan on the renal expression of nephrin and TGF-β1 in DN mice. Representative Western blots (A and C) showing specific bands for nephrin and TGF-β1, respectively, and GAPDH as an internal control and quantified by using densitometric analysis (B and D). An equal amount of protein sample obtained from kidney homogenate was applied to each lane. In DN mice, telmisartan treatment significantly reduced the protein expression of TGF-β1 and no change in the expression of nephrin. NG, vehicle only treated group; DG, STZ-induced diabetic group; TG, telmisartan (5 mg/kg BW) treated diabetic group. All values are expressed as the mean ± SEM; $n = 5$; $^{*}p < 0.05$ vs NG, $^{\#}p < 0.05$ vs DG.

with the DG mice (Figure 2), suggesting that the beneficial effect of telmisartan treatment in STZ-induced oxidative stress could be mainly by suppressing the NADPH oxidase system through the down-regulation of AT-1R.

Numerous studies have reported the involvement of Ang-II in fibrosis in STZ-induced DN. Activation of Ang-II in kidney would stimulate the protein synthesis in mesangial cells (MCs) and tubular cells [40,41], leading to hypertrophy and synthesis of extracellular components, mainly fibronectin [42,43], collagen and TGF-β [44,45] which are contributed to the pathogenesis of fibrosis [46]. We also found that the DG mice showed a significant increase in renal hypertrophy as well as the expressions of fibrosis markers, such as TGF-β1, fibronectin and collagen-III in comparison to NG mice, and the telmisartan treatment significantly attenuated the renal hypertrophy and those fibrotic markers in comparison to DG mice (Figures 4C and 6).

In addition, we could not rule out the possible involvement of PPAR-γ in all the beneficial effects of telmisartan, since it has been proved to be a partial agonist for PPAR-γ [47]. Furthermore, it has been demonstrated that telmisartan suppressed the AT-1R expression in both mRNA and protein levels through the PPAR-γ-mediated pathway [48]. In this study, we have already shown that telmisartan treatment would increase the protein expressions of ACE-2 as well as Ang-(1–7) *mas* receptor, which suggests that telmisartan might increase the PPAR-γ protein as well as its downstream protein PGC-1α expressions through the up-regulation of Ang-(1–7) *mas* receptor. Our findings are well supported by the fact that the treatment of Ang-(1–7) would prevent diabetes-induced attenuation of PPAR-γ in kidney [49]. Numerous studies have reported that the activation of PPAR-γ exerts anti-oxidative, vasculo-protective effects [50] and reduced NADPH oxidase sub-units protein expressions [51]. In addition to this, telmisartan has been proved to inhibit the renal fibrosis by attenuating TGF-β1 and the downstream production of ECM [52]. Now it is clearly evident that the telmisartan would lbe beneficial in reducing renal hypertrophy and fibrosis mainly through its antagonistic action of Ang-II as well as its PPAT-g agonist action in STZ-induced DN.

Furthermore, several studies have implicated the involvement of mitogen activated protein kinase (MAPK), including p38 MAPK in kidney diseases. A recent report states that the STZ injection would cause the significant activation of p38 MAPK due to the increased mRNA levels of MKK3 and MKK6 in diabetic glomeruli rats [53]. In addition, this increased p38 MAPK activity might play an important role in the development of early hypertrophy and ECM accumulation during STZ-induced DN [54]. Our results showed that the treatment of DN complications, such as fibrosis with telmisartan, might be effective, because the telmisartan could prevent the Ang-II mediated p38 MAPK activation, thereby it would provide the beneficial effects on fibrosis. Reddy et al. [55] reported that the treatment of Ang-II in rats could activate the p38 MAPK, leading to the

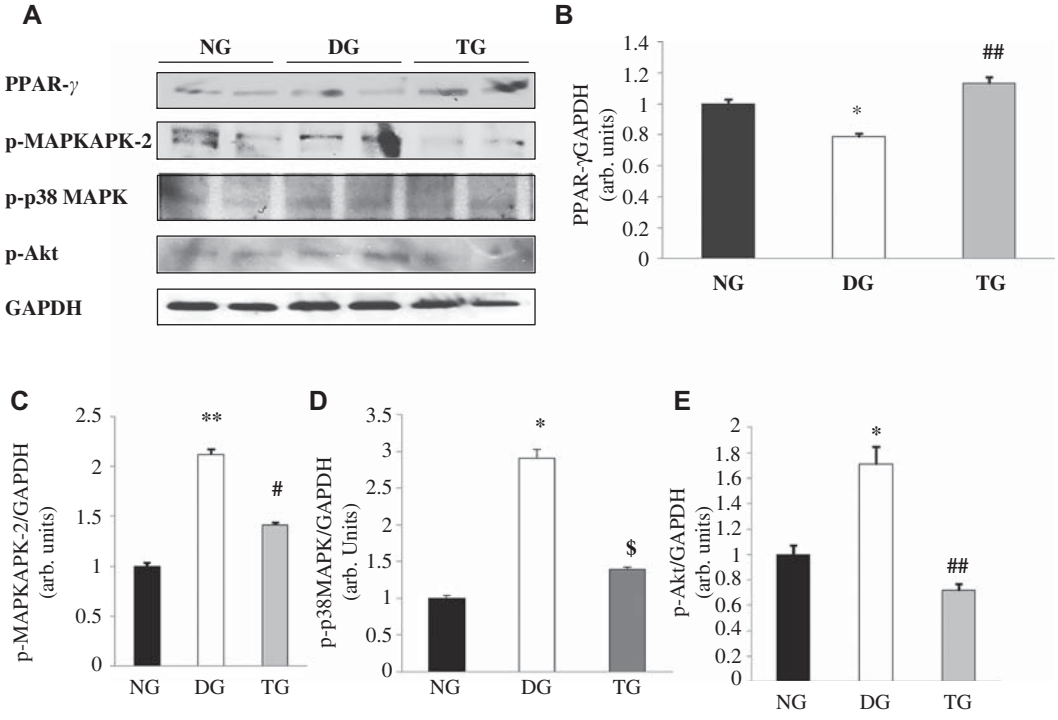

Figure 5. Effect of telmisartan on the renal expression of PPAR-γ, p-MAPKAPK-2, p-p38 MAPK and p-Akt in DN mice. Representative Western blots (A) showing specific bands for PPAR-γ, p-MAPKAPK-2, p-p38 MAPK and p-Akt, respectively, and GAPDH as an internal control and quantified by using densitometric analysis (B–E). An equal amount of protein sample obtained from kidney homogenate was applied to each lane. In DN mice, telmisartan treatment significantly reduced the renal phosphorylation of p-MAPKAPK-2, p38 MAPK and p-Akt and up-regulated the protein expression of PPAR-γ. NG, vehicle only treated group; DG, STZ-induced diabetic group; TG, telmisartan (5 mg/kg BW) treated diabetic group. All values are expressed as the mean ± SEM; $n = 5$; $^{*}p<0.05$ and $^{**}p<0.01$ vs NG, $^{\#}p<0.05$ and $^{\#\#}p<0.01$ vs DG. $^{\$}p<0.05$ vs NG.

induction of fibronectin expression in MCs. Oudit et al. [56] have reported that ACE-$2^{-/-}$ mice showed increased oxidative stress and inflammation, as well as MAP kinase activation, which indicates the relation between the RAS and MAPK indirectly. Furthermore, we already reported that the activation of p38 MAPK during STZ injection could activate its downstream effector p-MAPKAPK-2 in dominant negative p38α mice in heart [25]. Consistent with previous data, attenuation of p38 MAPK by telmisartan treatment,

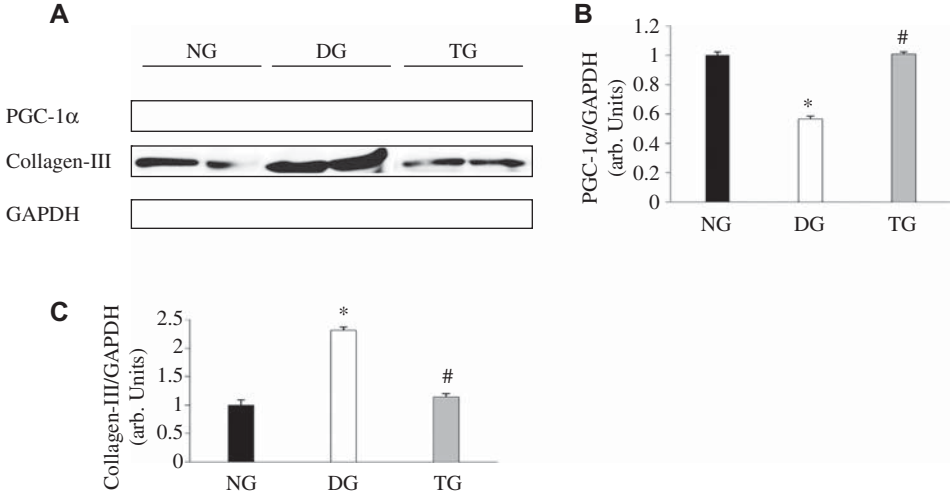

Figure 6. Effect of telmisartan on the renal expression of collagen-III and PGC-1α in DN mice. Representative Western blots (A) showing specific bands for collagen-III and PGC-1α, respectively, and GAPDH as an internal control and quantified by using densitometric analysis (B and C). An equal amount of protein sample obtained from kidney homogenate was applied to each lane. In DN mice, telmisartan treatment significantly reduced the renal collagen-III and up-regulated the expression of PGC-1α. NG, vehicle only treated group; DG, STZ-induced diabetic group; TG, telmisartan (5 mg/kg BW) treated diabetic group. All values are expressed as the mean ± SEM; $n = 5$; $^{*}p < 0.05$ vs NG. $^{\#}p < 0.05$ vs DG.

suggesting that it could inhibit the significant increase in collagen level as well as the increased expression of TGF-β1 in diabetic glomeruli which are known to mediated by the Ang-II. (Figure 5). Furthermore, studies focused on the involvement of Akt in diabetic complications suggest both increased as well as decreased activity in Akt. Zdychova and Komers [57] reported the reason for the variability in Akt is highly dependent on the type of diabetes as well as cell type. We found that the increased phosphorylation of Akt in DG mice was decreased significantly by telmisartan treatment [55]. Furthermore, in contrast, we did not observe any significant up-regulation or down-regulation in the protein expression of nephrin among the groups (Figure 4A). The reason for this activity is largely unknown.

Our results are consistent with previously reported data, suggesting that the modulation of Ang-II would play an important role in the development of DN through the down-regulation of ACE-2 dependent Ang-(1–7) *mas* receptor which in turn leads to an increased oxidative stress and fibrosis. Treatment with telmisartan might reduce the development of early DN, possibly by up-regulating the ACE-2 dependent Ang-(1–7) *mas* receptor associated with its PPAR-γ agonistic action.

Acknowledgement

We thank Sayaka Mito, Vijayakumaran Sukumaran, Wawaimuli Arozal, Yoshiyasu Kobayashi and Somasundaram Arumugam for their assistance in this research work.

Declaration of interest

This research was supported by a Yujin Memorial Grant, Ministry of Education, Culture, Sports and Technology of Japan and by a grant from the Promotion and Mutual Aid Corporation for Private Schools, Japan.

References

[1] Lasaridis AN, Sarafidis PA. Diabetic nephropathy and antihypertensive treatment: what are the lessons from clinical trials? Am J Hyper 2003;16:689–697.
[2] Molitch ME, DeFronzo RA, Franz MJ. Nephropathy in diabetes. Diabetes Care 2004;27:79–83.
[3] Mogensen CE, Keane WF, Bennett PH, Jerums G, Parving HH, Passa P. Prevention of diabetic renal disease with special reference to microalbuminuria. Lancet 1995;346: 1080–1084.
[4] Remuzzi G, Benigni A, Remuzzi A. Mechanisms of progression and regression of renal lesions of chronic nephropathies and diabetes. J Clin Invest 2006;116:288–296.
[5] Lane TA, Lamkin GE, Wancewicz EV. Protein kinase C inhibitors block the enhanced expression of intercellular adhesion molecule-1 of endothelial cells activated by interleukin-1, lipopolysaccharide and tumor necrosis factor. Biochem Biophys Res Commun 1990;103:172–177.
[6] Ibrahim HN, Hostetter TH. Diabetic nephropathy. J Am Soc Nephrol 1997;8:487–493.
[7] Wolf G, Ziyadeh FN. The role of Angiotensin II in diabetic nephropathy: emphasis on nonhemodynamic mechanisms. Am J Kidney Diseases 1997;29:153–163.
[8] Wolf G. Angiotensin II as a mediator of tubulointerstitial injury. Nephrol Dial Transplant 2000;15:61–63.
[9] Luft FC. Proinflammatory effects of angiotensin II and endothelin: targets for progression of cardiovascular and renal disease. Curr Opin Nephrol Hypertens 2002;11:59–66.
[10] Lee EA, Seo JY, Jiang Z, Yu MR, Kwon MK, Ha H, Lee HB. Reactive oxygen species mediate high glucose-induced plasminogen activator inhibitor-1 upregulation in mesangial cells and in diabetic kidney. Kidney Int 2005;67:1762–1771.
[11] Li JM, Shah AM. ROS generation by nonphagocytic NADPH oxidase: potential relevance in diabetic nephropathy. J Am Soc Nephrol 2003;14:221–226.
[12] Kanwar YS, Wada J, Sun L, Xie P, Wallner EI, Chen S, Chugh S, Danesh FR. Diabetic nephropathy: mechanisms of renal disease progression. Exp Biol Med 2008;233:4–11.
[13] Thallas-Bonke V, Thorpe SR, Coughlan MT, Fukami K, Yap FY, Sourris KC, Penfold SA, Bach LA, Cooper ME, Forbes JM. Inhibition of NADPH oxidase prevents advanced glycation end product-mediated damage in diabetic nephropathy through a protein kinase C-alpha-dependent pathway. Diabetes 2008;57:460–469.
[14] Parving HH, Lehner H, Brochner-Mortensen J. The effect of irbesartan on the development of diabetic nephropathy in patients with type 2 diabetes. N Engl J Med 2001;345:870–878.
[15] Nakao N, Yoshimura A, Morita H. Combination treatment of angiotensin-II receptor blocker and angiotensin converting-enzyme inhibitor in non-diabetic renal disease (COOPERATE): a randomized controlled trial. Lancet 2003;361:117–124.
[16] Sugiyama H, Kobayashi M, Wang DH, Sunami R, Maeshima Y, Yamasaki Y, Masuoka N, Kira S, Makino H. Telmisartan inhibits both oxidative stress and renal fibrosis after unilateral ureteral obstruction in acatalasemic mice. Nephrol Dial Transplant 2005;20:2670–2680.
[17] Klahr S, Morrissey J. Obstructive nephropathy and renal fibrosis. Am J Physiol Renal Physiol 2002;283:F861–F875.
[18] Satoh M, Kashihara N, Yamasaki Y. Renal interstitial fibrosis is reduced in angiotensin II type 1a receptor-deficient mice. J Am Soc Nephrol 2001;12:317–325.
[19] Seeland U, Schäffer A, Selejan S, Hohl M, Reil JC, Müller P, Rosenkranz S, Böhm M. Effects of ATI- and beta-adrenergic receptor antagonists on TGF-beta1-induced fibrosis in transgenic mice. Eur J Clin Invest 2009;39:851–859.
[20] Yao Y, Zou R, Liu X, Jian J, Huang Q, He Y, Li M, Wang S, Zhou J, Ma D, Xu G. Telmisartan but not valsartan inhibits TGF-beta mediated accumulation of extracellular matrix via activation of PPARgamma. E Pub 2008;28:543–548.
[21] Gurusamy N, Watanabe K, Ma M, Zhang S, Muslin AJ, Kodama M, Aizawa Y. Dominant negative 14-3-3 promotes cardiomyocyte apoptosis in early stage of type I diabetes mellitus through activation of JNK. Biochem Biophys Res Commun 2004;320:773–780.
[22] Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC. Measurement of protein using bicinchoninic acid. Anal Biochem 1985;150:76–85.
[23] Ibrahim MA, Ashour OM, Ibrahim YF, El-Bitar HI, Gomaa W, Abdel-Rahim SR. Angiotensin-converting enzyme inhibition and angiotensin AT(1)-receptor antagonism equally improve doxorubicin-induced cardiotoxicity and nephrotoxicity. Pharmacol Res 2009;60:378–381.
[24] Cosentino F, Hishikawa K, Katusic ZS. High glucose increases nitric oxide synthase expression and superoxide anion generation in human aortic endothelial cells. Circulation 1997;96:25.
[25] Thandavarayan RA, Watanabe K, Sari FR, Ma M, Lakshmanan AP, Giridharan VV, Gurusamy N, Nishida H,

Konishi T, Zhang S, Muslin AJ, Kodama M, Aizawa Y. Modulation of doxorubicin-induced cardiac dysfunction in dominant-negative p38α mitogen-activated protein kinase mice. Free Radic Biol Med 2010;49:1422–1431.

[26] Benson SC, Iguchi R, Ho CI, Yamamoto K, Kurtz TW. Inhibition of cardiovascular cell proliferation by angiotensin receptor blockers: are all molecules the same? J Hypertens 2008;26:973–980.

[27] Tipnis SR, Hooper NM, Hyde R, Karran E, Christie G, Turner AJ. A human homolog of angiotensin-converting enzyme: cloning and functional expression as a captopril-insensitive carboxypeptidase. J Biol Chem 2000;275:33238–33243.

[28] Singh R, Singh A, Leehey DJ. A novel mechanism for angiotensin II formation in streptozotocin-diabetic rat glomeruli. Am J Physiol Renal Physiol 2005;288:F1183–F1190.

[29] McCollum LT, Gallagher PE, Ferrario CM, Tallant EA. Angiotensin-(1-7) reduces COX-2 in cardiac fibroblasts. Hypertension 2006;48:79.

[30] Tikellis C, Johnston CI, Forbes JM, Burns WC, Burrell LM, Risvanis J, Cooper ME. Characterization of renal angiotensin-converting enzyme 2 in diabetic nephropathy. Hypertension 2003;41:392–397.

[31] Betul K, Melek O, Matem T, Omer U, Fatma KD, Itır Y, Ekrem E. Renoprotective effects of valsartan and enalapril in STZ-induced diabetes in rats. Acta Histochemica 2002;104:123–130.

[32] Etoh T, Inoguchi T, Kakimoto M, Sonoda N, Kobayashi K, Kuroda J, Sumimoto H, Nawata H. Increased expression of NAD(P)H oxidase subunits, NOX4 and p22phox, in the kidney of streptozotocin induced diabetic rats and its reversibility by interventive insulin treatment. Diabetologia 2003;46:1428–1437.

[33] Geiszt M, Lekstrom K, Witta J, Leto TL. Proteins homologous to p47phox and p67phox support superoxide production by NAD(P)H oxidase 1 in colon epithelial cells. J Biol Chem 2003;278:20006–20012.

[34] Cui XL, Douglas JG. Arachidonic acid activates c-jun N-terminal kinase through NADPH oxidase in rabbit proximal tubular epithelial cells. Proc Natl Acad Sci USA 1997; 94:3771–3776.

[35] Jones SA, Hancock JT, Jones OT, Neubauer A, Topley N. The expression of NADPH oxidase components in human glomerular mesangial cells: detection of protein and mRNA for p47phox, p67phox, and p22phox. J Am Soc Nephrol 1995;5:1483–1491.

[36] Lassegue B, Clempus RE. Vascular NAD(P)H oxidases: specific features, expression, and regulation. Am J Physiol 2003; 285:R277–R297.

[37] El-Benna J, My-Chan Dang P, Gougerot-Pocidalo M-A, Marie J-C, Braut-Boucher F. p47phox, the phagocyte NADPH oxidase/NOX2 organizer: structure, phosphorylation and implication in diseases. Exp Mol Med 2009;41:217–225.

[38] Onozato ML, Tojo A, Goto A, Fujita T, Wilcox CS. Oxidative stress and nitric oxide synthase in rat diabetic nephropathy: effects of ACEI and ARB. Kidney Int 2002;61:186–194.

[39] Wendt MC, Daiber A, Kleschyov AL, Mülsch A, Sydow K, Schulz E, Chen K, Keaney JF Jr, Lassègue B, Walter U, Griendling KK, Münzel T. Differential effects of diabetes on the expression of the gp91phox homologues nox1 and nox4. Free Radiac Biol Med 2005;39:381–391.

[40] Jaimes EA, Galceran JM, Raij L. Angiotensin II induces superoxide anion production by mesangial cells. Kidney Int 1998;54:775–784.

[41] Block K, Ricono JM, Lee DY, Bhandari B, Choudhury GG, Abboud HE, Gorin Y. Arachidonic acid dependent activation of a p22(phox)-based NAD(P)H oxidase mediates angiotensin II-induced mesangial cell protein syntheses and fibronectin expression via Akt/PKB. Antioxid Redox Signal 2006;8:1497–1508.

[42] Pearse DD, Tian RX, Nigro J, Lorgulescu JB, Puzis L, Jaimes EA. Angiotensin II increases the expression of the transcription factor ETS-1 in mesangial cells. Am J Physiol Renal Physiol 2008;294: F1094–F1100.

[43] Zhao W, Chen SS, Chen Y, Ahokas RA, Sun Y. Kidney fibrosis in hypertensive rats: role of oxidative stress. Am J Nephrol 2008;28:548–554.

[44] Lodha S, Dani D, Mehta R, Bhaskaran M, Reddy K, Ding G, Singhal PC. Angiotensin II-induced mesangial cell apoptosis: role of oxidative stress. Mol Med 2002;8:830–840.

[45] Aradaillou R, Chansel D, Chatziantoniou C, Dussaule JC. Mesangial AT1 receptors: expression, signaling, and regulation. J Am Soc Nephrol 1999;10:S40–S46.

[46] Rosen P, Nawroth PP, King G, Moller W, Tritschler HJ, Packer L. The role of oxidative stress in the onset and progression of diabetes and its complications: a summary of a Congress Series sponsored by UNESCOMCBN, the American Diabetes Association and the German Diabetes Society. Diabete Metab Res Rev 2001;17:189–212.

[47] Benson SC, Pershadsingh HA, Ho CI, Chittiboyina A, Desai P, Pravenec M, Qi N, Wang J, Avery MA, Kurtz TW. Identification of telmisartan as a unique angiotensin II receptor antagonist with selective PPARgamma-modulating activity. Hypertension 2004;43:993–1002.

[48] Imayama I, Ichiki T, Inanaga K, Ohtsubo H, Fukuyama K, Ono H, Hashiguchi Y, Sunagawa K. Telmisartan down regulates angiotensin II type 1 receptor through activation of peroxisome proliferator-activated receptor gamma. Cardiovasc Res 2006;72:184–190.

[49] Dhaunsi GS, Yousif MH, Akhtar S, Chappell MC, Diz DI, Benter IF. Angiotensin-(1-7) prevents diabetes-induced attenuation in PPAR-gamma and catalase activities. Eur J Pharmacol 2010;638:108–114.

[50] Tao L, Liu HR, Gao E, Teng ZP, Lopez BL, Christopher TA, Ma XL, Batinic-Haberle I, Willette RN, Ohlstein EH, Yue TL. Antioxidative, antinitrative, and vasculoprotective effects of a peroxisome proliferator-activated receptor-gamma agonist in hypercholesterolemia. Circulation 2003;108:2805–2811.

[51] Hwang J, Kleinhenz DJ, Rupnow HL, Campbell AG, Thulé PM, Sutliff RL, Hart CM. The PPARgamma ligand, rosiglitazone, reduces vascular oxidative stress and NADPH oxidase expression in diabetic mice. Vascul Pharmacol 2007; 46:456–462.

[52] Prasad B, Ravi N, Asma K, James RS, Adam WC. Angiotensin receptor blockers for the reduction of proteinuria in diabetic patients with overt nephropathy: results from the AMADEO study. Vasc Health Risk Manag 2009;5:129–140.

[53] Dunlop ME, Muggli EE. Small heat shock protein alteration provide a mechanism to reduce mesangial cell contractility in diabetes and oxidative stress. Kidney Int 2000;57:464–475.

[54] Kang S, Adler SG, Janine L, Natarajan R. p38 MAPK and MAPK 3/6 mRNA and activities are increased in early diabetic glomeruli. Kidney Int 2001;60:543–552.

[55] Reddy MA, Adler SG, Kim Y, Lanting L, Rossi J, Kang S, Nadler JL, Shahed A, Natarajan R. Interaction of MAPK and 12-lipooxygenase pathways in growth and matrix protein expression in mesangial cells. Am J Physiol Renal Physiol 2002; 283:F985–F994.

[56] Oudit GY, Kasseri Z, Patel MP, Chappell MC, Buany J, Backx PH, Tsushima RG, Scholey JW, Khokha R, Penninger JM. Angiotensin II oxidative stress and inflammation mediate the age dependent cardiomyopathy in ACE2 null mice. Cardiovascular Res 2007;75:29–39.

[57] Zdychova J, Komers K. Emerging role of akt kinase/protein kinase B signaling in pathophysiology of diabetes and its complications. Physiol Res 2005;54:1–16.

This paper was first published online on Early Online on 3 March 2011.