Neuronal Nitric Oxide Synthase and Cyclooxygenase-2 in Diabetic Nephropathy of Type 2 Diabetic OLETF Rats

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Abstract: Neuronal nitric oxide synthase (nNOS) and cyclooxygenase-2 (COX-2) regulate the tubuloglomerular feedback (TGF) and renin-angiotensin system (RAS) in the kidney. In type 1 diabetic rats, renal overproduction of these enzymes and their relationship to the pathogenesis of diabetic nephropathy has been demonstrated. In the present study, we histologically and immunohistochemically investigated the kidneys of Otsuka Long-Evans Tokushima Fatty (OLETF) rats, as a model of type 2 diabetes, at 62 weeks of age (chronic phase of diabetes). The kidneys of OLETF rats showed typical diabetic nephropathy. Quantitative scores for glomerulosclerosis and interstitial fibrosis in OLETF rats were significantly higher than those of age-matched control Long-Evans Tokushima Otsuka (LETO) rats. nNOS- and COX-2-positive immunoreactions were observed in the distal tubules and collecting ducts. These reactions appeared to be more widely distributed in OLETF, and the number of nNOS- and COX-2-positive sites in the OLETF were significantly more than those in LETO rats. Expression of renin, angiotensin II, and inducible nitric oxide synthase (iNOS) were also examined immunohistochemically, and no differences between OLETF and LETO rats were observed in the distributions and the number of immunoreactive-sites. In conclusion, the overproduction of nNOS and COX-2 in the kidney of OLETF rats was confirmed, suggesting that the overproduction of nNOS and/or COX-2 does not affect the intrarenal RAS or iNOS production but does affect TGF.

Key words: cyclooxygenase-2, nephropathy, nitric oxide synthase, tubuloglomerular feedback, type 2 diabetes

Introduction

The Otsuka Long-Evans Tokushima Fatty (OLETF) rat is an established model of human type 2 diabetes. This model develops a diabetic syndrome in nearly 100% of male rats at 25 weeks of age and hyperglycemia and hyperinsulinemia are exhibited in the early phase of the disease as a result of islet cell hyperplasia
and peripheral insulin resistance [12, 13, 27]. As age proceeds, the rat eventually develops hypoinsulinemia as a result of deterioration of the islets beta cells, and pathological changes in the kidneys occur from 40 weeks of age [13]. Pathological changes of the kidneys in OLETF rats include severe glomerulosclerosis and tubulointerstitial fibrosis similar to those in human diabetic nephropathy [6, 13–15, 21].

The macula densa (MD) is a specialized region of the distal straight tubule (thick ascending limb) adjacent to the vascular pole of the glomerulus. This region controls renin-secretion from juxtaglomerular cells of the afferent glomerular arteries and the tubuloglomerular feedback (TGF) system via detection of changes in Cl– concentration in the luminal fluid [7, 22]. These mechanisms are regulated by two enzymes: neuronal nitric oxide synthase (nNOS) and cyclooxygenase-2 (COX-2), and both enzymes are primarily expressed in MD cells and stimulate renin-secretion and TGF-inhibition [2, 8, 9, 23, 32]. In recent studies, increases in nNOS and COX-2 expression and their involvement in the pathogenesis of diabetic nephropathy were demonstrated in streptozotocin (STZ)-induced type 1 diabetic rats [1, 16–18, 25, 28]. On the other hand, although most diabetes in adult humans is type 2 diabetes, renal expression of these enzymes and their involvement in the pathogenesis of diabetic nephropathy remains unresolved in type 2 diabetes. The OLETF rat is a good model of diabetic nephropathy in human type 2 diabetes, as described above. Therefore, in the present study, we histologically and immunohistochemically investigated the kidneys of 62-week-old OLETF rats (chronic phase of diabetes) to assess the expression of nNOS, COX-2 and other related proteins in diabetic nephropathy.

**Materials and Methods**

**Animals**

Age-matched male OLETF (n=5) and control Long-Evans Tokushima Otsuka (LETO) rats (n=5) were bred and housed in an animal room (temperature, 23 ± 2°C; humidity, 60 ± 10%; lighting cycle, 12 h; ventilation 13–15 cycles/h) at the Otsuka Pharmaceutical Co. Ltd., Tokushima, Japan. Animals were given a commercial diet containing 22.4% crude protein/100 g (CRF-1; Oriental Yeast, Tokyo, Japan) and tap water *ad libitum*. Onset of diabetes was confirmed by an oral glucose tolerance test at 35 weeks of age [15].

**Tissue preparation**

All animals were sacrificed at 62 weeks of age under deep anesthesia using pentobarbital. They were perfused through the left ventricle with a physiological saline followed by Zamboni’s solution. After immersion fixation with the same solution, central slices of the kidneys were routinely embedded in paraffin. Sections, 3 µm thick, were selected every 30 µm and stained with hematoxylin-eosin (HE), periodic acid Schiff (PAS), periodic acid methenamine-silver (PAM) and Masson’s trichrome (MT).

**Quantitative histology**

Sections stained with MT (3 sections/animal) were quantitatively analyzed at random as follows. The extent of glomerulosclerosis was evaluated using a semiquantitative scoring system described previously [26]. Briefly, approximately 100 glomeruli per animal were examined, and the severity of the sclerotic lesions was graded from 0 to +4, where 0 = normal, +1 = up to 25% sclerosis, +2 = 26 to 50% sclerosis, +3 = 51 to 75% sclerosis, and +4 = 76 to 100% sclerosis. The score of glomerulosclerosis for each animal was evaluated. If, for example, 10 of the 100 glomeruli displayed +1 lesions, 20 of the 100 demonstrated +2 lesions, 15 of the 100 demonstrated +3 lesions and five of the 100 demonstrated +4 lesions, the final sclerosis score would be: \( \frac{1 \times 10}{100} + \frac{2 \times 20}{100} + \frac{3 \times 15}{100} + \frac{4 \times 5}{100} \) \times 100 = 115. The extent of interstitial fibrosis was also evaluated using a similar semiquantitative scoring system. Briefly, approximately 20 non-overlapping cortical fields (magnification × 200) per animal were examined, and the severity of fibrotic lesions was graded from 0 to +4: 0 = normal, +1 = mild fibrosis, +2 = moderate fibrosis, +3 = severe fibrosis, +4 = very severe fibrosis. The score of interstitial fibrosis for each animal was evaluated. If, for example, five of the 20 fields displayed +1 lesions, three of the 20 demonstrated +2 lesions, two of the 20 demonstrated +3 lesions, and one of the 20 demonstrated +4 lesions, the final fibrosis score would be: \( \left(\frac{1 \times 5}{20}\right) + \left(\frac{2 \times 3}{20}\right) + \left(\frac{3 \times 2}{20}\right) + \left(\frac{4 \times 1}{20}\right) \) \times 100 = 105.

**Immunohistochemistry**

Immunohistochemistry was performed using an *Elite*
ABC kit (Vector Laboratories, Burlingame, CA, USA). Anti-nNOS polyclonal antibody (Cayman Chemical, Ann Arbor, MI, USA) diluted 1:1,500, anti-COX-2 polyclonal antibody (Cayman Chemical) diluted 1:800, anti-angiotensin II polyclonal antibody (Peninsula Laboratories, San Carlos, CA, USA) diluted 1:800, anti-iNOS polyclonal antibody (Lab Vision Corporation, Fremont, CA, USA) diluted 1:100 or anti-renin polyclonal antibody (supplied by Dr. Murakami, University of Tsukuba) diluted 1:10,000 were used as primary antibodies. Incubation with the primary antibodies was performed overnight at 4°C. Negative control sections were incubated with non-immunized rabbit IgG (Dako Cytomation, Glostrup, Denmark) instead of the primary antibody. Biotinylated goat anti-rabbit IgG (Vector Laboratories) diluted 1:200 was used as the secondary antibody. Immunohistochemical reactivity was detected by a 0.025% (W/V) 3,3′-diaminobenzidine-0.003% (V/V) H₂O₂ solution. Immunoreactions were stopped in distilled water, and then sections were counterstained with Mayer’s hematoxylin. For antigen retrieval, all pre-treatments were performed after deparaffinization. For nNOS, angiotensin II or iNOS detection, sections were microwaved in a 10 mM citrate buffer (pH 6.0). For COX-2 detection, sections were microwaved in the same buffer and then treated with 0.3% Triton X-100 in 10 mM phosphate buffered saline. For renin detection, antigen retrieval was not required. The numbers of nNOS-, COX-2-, renin- or angiotensin II-positive sites were counted in approximately 20 non-overlapping cortical fields (magnification × 200) per animal.

Statistics
All quantitative results were expressed as the mean ± standard error (S.E.), and analyzed statistically using the Mann-Whitney U test.

Results
Histopathology
In control LETO rats, mild thickening of the basement membrane was commonly observed (Fig. 1a). In OLETF rats, glomerular and tubulointerstitial damages were apparently more severe than those in LETO rats. Glomerular damage characterized by the proliferation of the mesangial cells and endothelial cells, severe sclerosis with expansion of the mesangial matrices, and nodular lesions were observed in the kidneys of OLETF rats (Fig. 1b). Tubulointerstitial damage characterized by mononuclear infiltration into the interstitium, tubular atrophy with a thickening of the basement membranes, an increase in the interstitial connective tissues, and urinary casts were also severe in the OLETF rats (Fig. 1b). The semiquantitative scores for glomerulosclerosis and interstitial fibrosis in OLETF rats were significantly higher than those in the control LETO rats (Fig. 2a, b).
Immunohistochemistry

NOS: In control LETO rats, nNOS-positive reactions were commonly observed in the MD cells of the distal tubules, medullary collecting ducts, and podocytes of the glomeruli (Fig. 3a). In OLETF rats, expansion of the nNOS-positive area was observed in the distal tubules, and positive reactions were not limited to MD cells but were also observed in the cells around the MD regions (Fig. 3b). Furthermore, nuclei in the atrophic tubules (distal convoluted or cortical collecting regions) of OLETF rats showed clear positive reactions (Fig. 3c). Medullary collecting ducts and glomerular podocytes also showed stronger reactions in OLETF than in LETO rats. There were no immunoreactions in the negative control sections (Fig. 3d). The number of nNOS-positive sites in the OLETF rats was significantly higher than in LETO rats (Fig. 2c). The iNOS-immunohistochemical analysis showed positive reactions in distal tubules and podocytes both in OLETF and LETO rats, but no differences were found in their reactive intensity or localization (Fig. 4).

COX-2: In control LETO rats, COX-2-positive reactions were sporadically observed in the distal tubules, principally in the MD cells (Fig. 5a). Although cortical collecting ducts showed sporadic positive reactions, their staining intensity was clearly weaker than that of distal tubules. In addition, interstitial cells in the tip of papilla also showed positive reactivity. In OLETF rats, expansion of COX-2-immunoreactivity was remarkable in the distal tubules, and positive reactions were commonly observed not only in the MD but also in the non-MD regions (Fig. 5b). Immunoreactivity in other regions did not differ between OLETF and LETO rats. The number of COX-2-positive sites in the OLETF rats was significantly higher than in LETO rats (Fig. 2d).

Renin: Renin-positive reactions were commonly detected in juxtaglomerular cells of the afferent glomerular arteries in all animals, but no differences between OLETF and LETO rats were observed in their reactive intensity or localization (Fig. 6a, b). The number of renin-positive sites in OLETF rats did not differ significantly from that in LETO rats (Fig. 2e).

Angiotensin II: Angiotensin II-positive reactions were sporadically observed in afferent arteries, principally in
Fig. 3. Immunohistochemical detection of nNOS in the cortex: (a) LETO rats, (b, c) OLETF rats, and (d) negative control section from OLETF rat. In LETO rats, positive-immunoreactions in the distal tubules are limited to the macula densa regions (a). In OLETF rats, nNOS-positive cells are observed not only in the macula densa cells but also in cells around the macula densa regions (b). Arrows indicate macula densa regions (a&b). Several nuclei of the atrophic tubules in OLETF rats show clear positive-immunoreactions (c). Incubation with non-immunized IgG shows no immunoreactions in the OLETF rat kidneys including atrophic lesions (d). Bars: 100 µm.

Fig. 4. Immunohistochemical detection of iNOS in the cortex: (a) LETO rats, and (b) OLETF rats. Immunoreactions are observed in the distal tubular epithelium and glomerular podocytes of both animals. Arrows indicate immunoreactions in the podocytes. Bars: 100 µm.
the regions close to the vascular pole, and interstitial cells. These findings were common to all animals, and no differences between OLETF and LETO rats were observed in their reactive intensity or localization (Fig. 6c, d). The number of angiotensin-II-positive sites in OLETF rats did not differ significantly from that in LETO rats (Fig. 2f).
Nitric oxide synthesized by nNOS in the MD cells increases the glomerular filtration rate (GFR) via inhibition of the TGF system [32]. Recently, reports an increase in nNOS production in the kidneys of STZ-treated type 1 diabetic rats has been reported [17, 28], and it was demonstrated that this increase in intrarenal nNOS production in STZ-induced diabetes is directly affected by high blood glucose and osmolality [10]. Furthermore, Ito et al. [13] demonstrated that administration of 7-nitro indazole, a selective inhibitor of nNOS, in STZ-treated rats induces significant reduction of GFR.

In contrast to findings from STZ-induced type 1 diabetes, a decrease in intrarenal nNOS production involving GFR reduction was reported in obese Zucker rats, a model of type 2 diabetes [5]. However, that report concluded that the renal pathological features of obese Zucker rats did not resemble those of diabetic nephropathy in humans; thus, causes of changes in nNOS production in the kidneys of type 2 diabetes patients remain uncertain. The OLETF rat is a spontaneous model of type 2 diabetes that shows typical features of diabetic nephropathy including glomerulosclerosis [6, 14, 15], tubulointerstitial fibrosis [21], and hyperfiltration [30], which resemble human features. Histopathological changes start to develop from 22 weeks of age [6], and increases in GFR occur at 5 and 10 months of age [30]. In the present study using 62-week-old OLETF rats, typical diabetic nephropathy was confirmed histopathologically and quantitatively. Immunohistochemical analysis of nNOS demonstrated an expansion of positive areas, and an increase in immunoreactivity was apparent in the kidneys of OLETF rats. Also the number of nNOS-positive sites in OLETF rats was significantly higher than that in age-matched control LETO rats (Fig. 2c). These results demonstrate that intrarenal production of nNOS increased in the OLETF rats that suffered from chronic diabetes, and this overproduction of nNOS was likely associated with the development of the diabetic renal hyperfiltration.

Interestingly, nuclei in interstitial cells and atrophic tubular cells in OLETF rats showed clear positive reactions for nNOS. Translocation of nNOS into the nucleus was previously reported only in cultured cerebral cortical astrocytes, and regulation of iNOS gene transcription was suggested as the main function of nNOS translocation [34]. Although iNOS is constitutively expressed in the renal medulla [20], its functional role in the kidney is largely unknown [24]. Furthermore, involvement of renal iNOS production in the pathogenesis of diabetic nephropathy has not been demonstrated in previous studies using STZ-treated rats [31] and KK/Ta mice, a model of type 2 diabetes [29]. For the present study, we hypothesized that nNOS translocation observed in the OLETF rats might be related to regulation of iNOS production and therefore performed immunohistochemical detection of iNOS. However, localization or reactive intensity of iNOS-positive sites in the kidneys of OLETF rats did not differ from those of control LETO rats; thus, no correlation between nNOS translocation and iNOS production regulation was found in the present study.

Recent reports using STZ-induced type 1 diabetic rats demonstrated an increase in intrarenal COX-2 production in diabetic nephropathy and an inhibition of renal hyperfiltration after selective COX-2 inhibitor administration [1, 16, 18, 25]. In type 2 diabetes, an increase in COX-2 protein level was demonstrated in the renal microvessels of obese Zucker rats [4]. In the present study, although a significant increase of COX-2-positive sites was observed in the OLETF rat kidneys (Fig. 2d), the regions that showed COX-2 overproduction were not in the blood vessels but in the distal tubules and the collecting ducts (Fig. 5b). Such COX-2 overproduction in the kidneys of OLETF rats resembles that reported in STZ-treated rats [18].

Stimulation of renin release from juxtaglomerular cells has been considered a principal physiological function of intrarenal COX-2 [2, 8, 9]. Decreases in blood pressure and renal renin mRNA expression after selective COX-2 inhibitor administration were demonstrated in renovascular hypertensive model rats [33]. However, a recent experiment using STZ-induced diabetic rats demonstrated that COX-2-derived metabolites did not modulate renin production in diabetic nephropathy [19]. The present results support those of Komers et al. [19] and demonstrate that the distribution and number of renin- or angiotensin II-positive sites did not differ between OLETF and control LETO rats. Down-regulation of the TGF system was recently proposed to play a critical role in intrarenal COX-2 activity, which interacts with nNOS function. Ichihara et al. [11] re-
ported that during acute activation of the TGF system by acetazolamide, carbonic anhydrase inhibitor, up-regulation of COX-2 activity in response to nNOS activation buffers TGF-mediated afferent arterial constriction. Deng et al. [3] demonstrated that during acute activation of the TGF system by treatment with benzolamide, carbonic anhydrase inhibitor, administration of selective COX-2 inhibitor prevented TGF activation, a result which was identical to the effect of nNOS inhibition. In addition, we found that changes in intrarenal angiotensin II could not explain the prevention of TGF activation. These reports and the present findings suggest that intrarenal production of COX-2 increases in the kidneys of OLETF rats, and this over-production of COX-2 participates in the inhibition of the TGF system.

In conclusion, OLETF rats with diabetic nephropathy showed an increase in intrarenal nNOS and COX-2 proteins. These increases in nNOS and COX-2 did not involve changes in intrarenal immunoreactions for iNOS, renin, or angiotensin II. These findings suggest that increased production of nNOS or COX-2 in the kidneys suppresses the TGF system, and this suppression might be a critical mechanism for the development of diabetic renal hyperfiltration.

References

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