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Dysregulation of nitric oxide synthase activity and Bcl-2 and caspase-3 gene expressions in renal tissue of streptozotocin-induced diabetic rats

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Aim: To evaluate the effects of diabetes on nitric oxide synthase (NOS) activity and on gene expressions of B-cell lymphoma protein 2 (Bcl-2) and caspase-3 in the renal tissues of streptozotocin (STZ)-induced rats.

Materials and methods: Twenty rats were analyzed in 2 equally distributed groups of healthy controls and STZ-induced diabetic rats. Rats with blood glucose levels of ≥250 mg/dL were considered diabetic. Renal tissue sections were analyzed histomorphologically and immunohistochemically. Gene expressions of caspase-3 and Bcl-2 were evaluated by real-time quantitative polymerase chain reaction.

Results: Bcl-2 expression significantly decreased in the diabetic group, both at the gene and protein level. Meanwhile, the expression of caspase-3 increased in the diabetic group, especially in proximal renal tubule cells. However, endothelial NOS (eNOS) protein expression in the glomerular endothelial nuclei was stronger in the control group, and protein expression of inducible NOS (iNOS) increased in the renal medullar area in the diabetic group.

Conclusion: According to our results, hyperglycemia inhibits eNOS, leading to reduced nitric oxide production in endothelial cells. Inhibition of eNOS was correlated with glomerular cell loss due to apoptosis. Furthermore, increased expression of iNOS was associated with endothelial dysfunction in STZ-induced diabetic rats. The structure most resistant to diabetes-induced damage was the glomerulus, while the renal medullar and proximal renal tubule cells displayed damage and cicatrization.

Key words: Diabetes mellitus, iNOS, eNOS, Bcl-2, caspase-3

Introduction

Diabetes mellitus (DM) is a multisystemic disorder with many different factors involved in its etiology, including autoimmune mechanisms, genetic factors, and viral infections. At the onset of DM, the kidneys begin to grow and the glomerular filtration rate increases. Some time later, structural changes can occur in the glomerulus, which then form the basis of progressive diabetic nephropathy (DN) (1,2). Several pathways are known to be involved in the pathogenesis of DM. Two hypotheses have been proposed to explain the changes in early DN, which are the vascular (3) and tubular hypotheses (1). Both mechanisms show that hyperfiltration occurs in the early stages of DN and causes an increase in glomerular pressure, which further contributes to the sclerotic process of the glomeruli (4). Although the development of early-stage DN has been shown in several animal models, to our knowledge, a rodent model that demonstrates the advanced stage of DN has yet to be described (5). Intrarenal hemodynamic abnormalities, as manifested by glomerular hyperfiltration, are thought to be among the foremost factors responsible for the onset and progression

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of diabetic glomerulopathy (1). Nitric oxide (NO), which is synthesized by the nitric oxide synthase (NOS) enzyme, is one of the most important intra- and intercellular signaling molecules. Numerous studies have indicated that the renal vasculature is under the continuous and strong influence of NO. It has been proposed that during the increase of renal perfusion pressure, NO is released to compensate the autoregulatory process (6). NO also acts as a potent modulator of renal function and controls both afferent and efferent vascular tone, medullary blood flow, and glomerular ultrafiltration coefficient (7,8). Endothelial NOS (eNOS) is closely associated to the cell membrane and localized to the caveolae. eNOS is predominantly expressed in the macrovascular endothelium and along the renal microvascular tree. eNOS-driven basal release of NO from endothelial cells contributes to the maintenance of the normal vasodilatory tone. Under conditions of hyperglycemia and the associated increase of advanced glycation end products, excessive generation of superoxide and its interaction with NO results in the formation of peroxynitrites. These oxidize tetrahydrobiopterin, an important cofactor involved in normal eNOS activity. Relatively little is known about the contribution of inducible NOS (iNOS) to the total NO pool under diabetic conditions. According to our hypothesis, iNOS-driven NO release might also contribute to an increased NO pool, the excess of which in turn results in renal degenerations. It is also likely that under hyperglycemic conditions, increased formation of reactive oxygen species (ROS) might occur (8). Besides this, apoptosis also plays a crucial role in DM, especially mitochondria-associated signaling (9). For example, the B-cell lymphoma protein 2 (Bcl-2) protein family members are intrinsic pathway operators of apoptosis and control the release of cytochrome c and other intermembrane mitochondrial proteins to the cytosol. Members of the protease family that are collectively called caspases are also involved in the apoptotic process and their appearances vary depending on the cell type and stimulus. Finally, cell-cell interactions also have an important impact on renal tissue integrity. Macro- and microangiopathy, including accelerated atherosclerosis and nephropathy, are the most common complications of diabetes. The main goals of this study therefore were 1) the evaluation of eNOS and iNOS protein expression levels in an experimental diabetic animal model, and 2) the revelation of diabetes-induced alterations of apoptosis in renal tissues.

Materials and methods

Animals and experimental design

The study protocol complies with the European Community Guidelines for the use of experimental animals. All experiments were approved by the local Animal Ethics Committee at the Ege University School of Medicine. Twenty *Rattus albinus* male rats weighing 200-250 g each were selected for this research and studied in 2 equally distributed groups as follows: Group 1, healthy controls; Group 2, diabetic rats. All rats received a standard powdered diet and water, and they were housed under normal conditions with a 12-h light/dark cycle at 21 ± 1 °C with 30% humidity. Diabetes was induced by a single intraperitoneal injection of streptozotocin (STZ) at 55 mg/kg body weight freshly dissolved in 0.1 mol/L sodium citrate buffer (pH 4.7) (2). After 48 h from the administration of STZ, the tail vein blood glucose level was measured with a Bayer Contour® glucometer (Bayer AG, Leverkusen, Germany), and the animals with blood glucose levels of ≥250 mg/dL were considered diabetic (10-13).

Immunohistochemical staining

At the end of the observational and experimental time of 60 days, the animals were sacrificed. Their renal tissues were removed, fixed (Sigma Chemical Co., St Louis, Missouri, USA), and embedded in paraffin blocks. Cross-sections 5 μm thick were prepared (Leica RM 2145, Leica, Solms, Germany), dewaxed, and rehydrated through a graded ethanol series. After washing them with distilled water and a phosphate buffered saline (PBS) solution for 10 min, they were treated with 2% trypsin (Sigma Chemical Co.) in 50 mM Tris buffer (pH 7.5) at 37 °C for 15 min. Sections were delineated with a Dako pen (Dako, Glostrup, Denmark) and incubated in a solution of 3% H_2O_2 for 15 min to inhibit endogenous peroxidase activity, followed by incubation with the primary antibody directed against iNOS (Zymed, South San Francisco, California, USA; diluted 1:100), eNOS (Zymed; diluted 1:1000), Bcl-2 (BioVision

Inc., San Francisco, California, USA; diluted 1:100), or caspase-3 (BioVision Inc.; diluted 1:100). Sections were then incubated with biotinylated secondary antibody and streptavidin-conjugated horseradish peroxidase (both from the Zymed Histostain-plus-Peroxidase-kit, 85-9043, prepared according to manufacturer's instructions) for 30 min each. Finally, after incubation with diaminobenzidine (DAB; from the DeadEnd Colorimetric TUNEL system, Promega, Madison, Wisconsin, USA; prepared according to manufacturer's instructions) for 5 min, immunolabeling was revealed. Washes between all stages and antibody dilutions were performed with PBS. Sections were counterstained with Mayer's hematoxylin (Zymed), washed with tap water, dehydrated through a graded ethanol series, cleared in xylene, and mounted with Entellan (Merck, Darmstadt, Germany). Negative control samples were processed as described above, except that primary antibodies were omitted and replaced with PBS alone. Immunoreactivity of iNOS, eNOS, Bcl-2, and caspase-3 was assessed by light microscopy using an Olympus BX-51 microscope and Olympus C-5050 digital camera (Olympus Corporation, Tokyo, Japan).

Gomori's trichrome staining

The 5-μm-thick cross-sections taken from the renal tissues that were embedded in paraffin blocks were used in the deparaffinization stage. After successive staining with Chromotrope 2R modified Gomori trichrome stain (Sigma-Aldrich, Munich, Germany), phosphotungstic acid (Merck), and fast green (BDH Chemicals Ltd., Poole, Dorset, UK) solutions, they were rehydrated in a graded ethanol series, cleared in xylene (Carlo Erba, Milan, Italy), and mounted with Entellan (Merck). Sections were assessed by light microscopy using an Olympus BX-51 microscope and Olympus C-5050 digital camera (Olympus Corporation).

Evaluation of glomerular size

Two nonconsecutive sections of renal tissues per animal were examined histomorphometrically at ×20 original magnification (scale bar = 125 μm) by using Image-Pro Express Image Software (Image-Pro Express v.4.5, Media Cybernetics Inc., Bethesda, Maryland, USA). Measurements of the glomerular diameter were performed on a bilateral visceral epithelium of 50 glomeruli. The average length of these bisections represented the diameter of each glomerulus. The average glomerular diameter of the 50 glomeruli per section was recorded. Measurements were performed by 2 independent researchers who were blind to the experimental groups. Sections were randomly chosen in all groups.

RNA preparation and real-time quantitative reverse transcription-polymerase chain reaction

Total RNA was prepared from 50 mg of renal tissue that was immediately taken after rats were sacrificed, frozen in liquid nitrogen, and stored at –86 °C by using the TriPure Isolation Reagent (Roche Applied Science, Penzberg, Germany). A 10-μg quantity of total RNA was reverse transcribed with the Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science). Primer and probe sets were chosen from the Universal Probe Library (Roche Applied Science). Real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis of Bcl-2 and caspase-3 was performed on the LightCycler v.1.5 instrument (Roche Applied Science). Relative ratios were calculated by normalizing gene expression levels of each sample with the house-keeping gene glucose 6-phosphate dehydrogenase (G6PDH), achieved by using different G6PDH standards of known concentrations that were included in each run.

Statistical analysis

The variable n represents the number of animals used. Descriptive statistics (mean ± standard deviation) were calculated using SPSS 15.0 (SPSS, Chicago, Illinois, USA). Student's t-test was used to compare the groups of continuous data for significant differences. $P < 0.05$ was considered to indicate statistical significance.

Results

At the end of our study, we found that eNOS expression in the glomerular endothelial cells and the visceral and parietal capsule was stronger in the controls when compared to the diabetic group (Figures 1A and 1B). Our immunohistochemical findings also suggest that iNOS expression slightly increased in the proximal renal tubule cells of diabetic rats. Although the expression level of iNOS in glomerular endothelial cells and the visceral and parietal capsule were similar between the control and diabetic

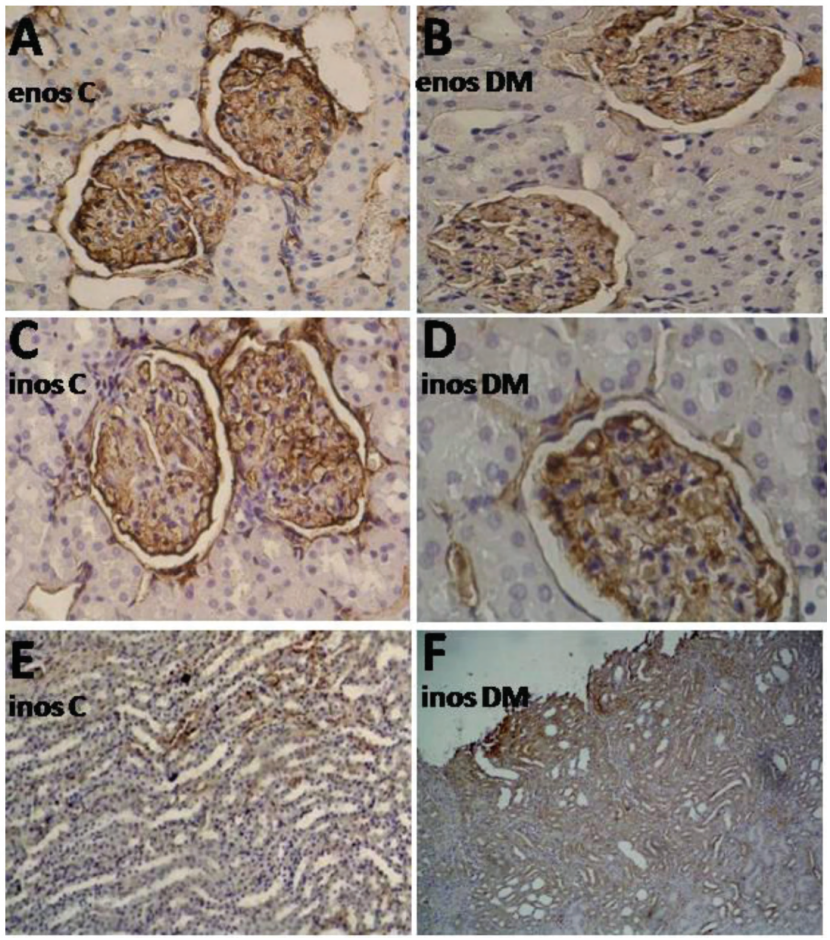

Figure 1. eNOS and iNOS immunostaining in renal tissue: A) Control group, renal cortex, eNOS immunohistochemical expression, magnification ×20; B) Diabetes group, renal cortex, decreased immunohistochemical expression of eNOS when compared to the control group (A), magnification ×20; C) Control group, renal cortex, iNOS immunohistochemical expression, magnification ×20; D) Diabetes group, renal cortex, similar immunohistochemical expression of iNOS in glomerular endothelial cells and visceral and parietal capsule when compared to the control group (C), magnification ×40; E) Control group, renal medulla, iNOS immunohistochemical expression, magnification ×10; F) Diabetes group, renal medulla, increased immunohistochemical expression of iNOS when compared to the control group (E), magnification ×4.

groups (Figures 1C and 1D), its expression was again increased in the renal medullar area of diabetic rats (Figures 1E and 1F). Immunohistochemical analyses of the investigated apoptotic proteins showed that while the expression of Bcl-2 decreased (Figures 2 A and 2B), the expression of caspase-3 increased in the diabetic group, especially in proximal renal tubule cells (Figures 2C and 2D). Staining with Gomori's trichrome revealed focal, chronic mononuclear inflammatory cell reaction, together with fibroblastic and cicatrized parts of the renal medullar tissue of diabetic rats as compared to the control (Figures 2E and 2F). The glomerular diameter was compared between the control and diabetic groups in order to analyze whether the glomeruli were spherical or showed any other significant morphological changes, and to evaluate vasodilatation and vasoconstriction, but no statistically significant difference could be observed (Figure 3A; Table).

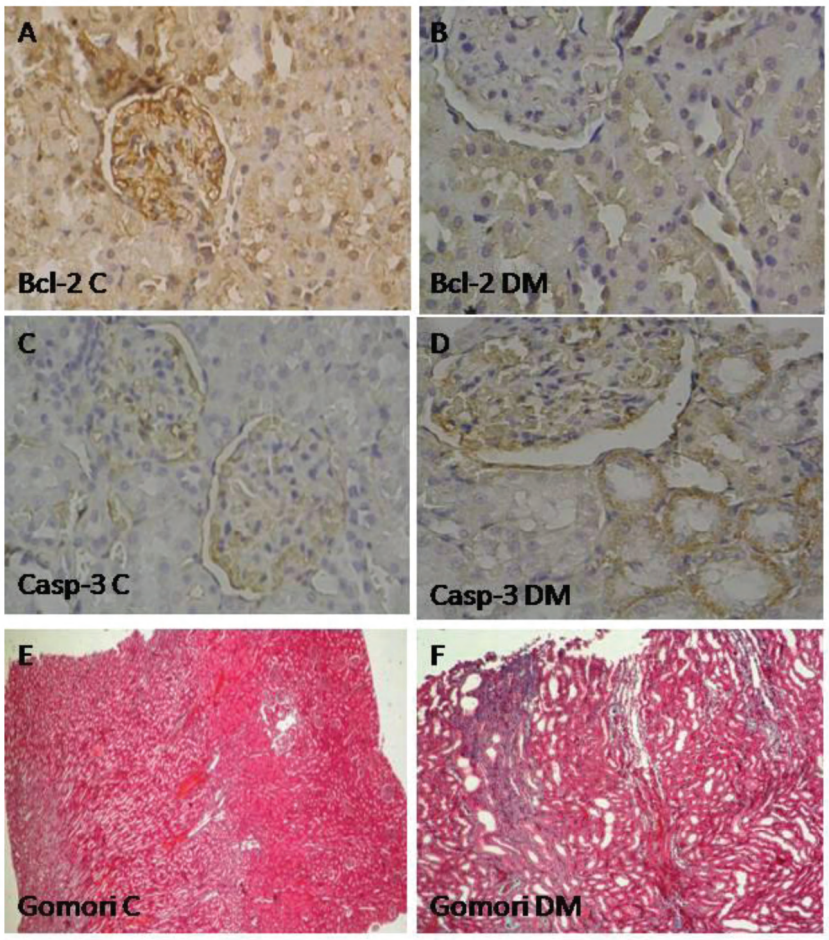

Figure 2. Bcl-2 and caspase-3 immunostaining together with Gomori's trichrome staining in renal tissue: A) Control group, renal cortex, Bcl-2 immunohistochemical expression, magnification ×20; B) Diabetes group, renal cortex, decreased immunohistochemical expression of Bcl-2 when compared to the control group (A), magnification ×40; C) Control group, renal cortex, caspase-3 immunohistochemical expression, magnification ×40; D) Diabetes group, renal cortex, increased immunohistochemical expression of caspase-3 especially in proximal renal tubule cells when compared to the control group (C), magnification ×40; E) Control group, renal medulla, Gomori's trichrome staining, magnification ×4; F) Diabetic group, renal medulla, Gomori's trichrome staining, apparent chronic mononuclear inflammatory cell reaction in renal medullar tissue when compared to the control group (E), magnification ×10.

Similar to the protein expression level determined by immunohistochemistry, the gene expression level of Bcl-2 significantly decreased in the diabetic group when compared with the controls (P = 0.03, Figure 3B). Caspase-3 protein and gene expression results did not correlate well with each other. When immunohistochemically analyzed, the expression level of caspase-3 increased in the diabetic group, but according to gene expression analyses the caspase-3 level did not significantly change between the diabetic and control groups (P = 0.559, Figure 3C). Gene expression levels of Bcl-2 and caspase-3 are shown in the Table.

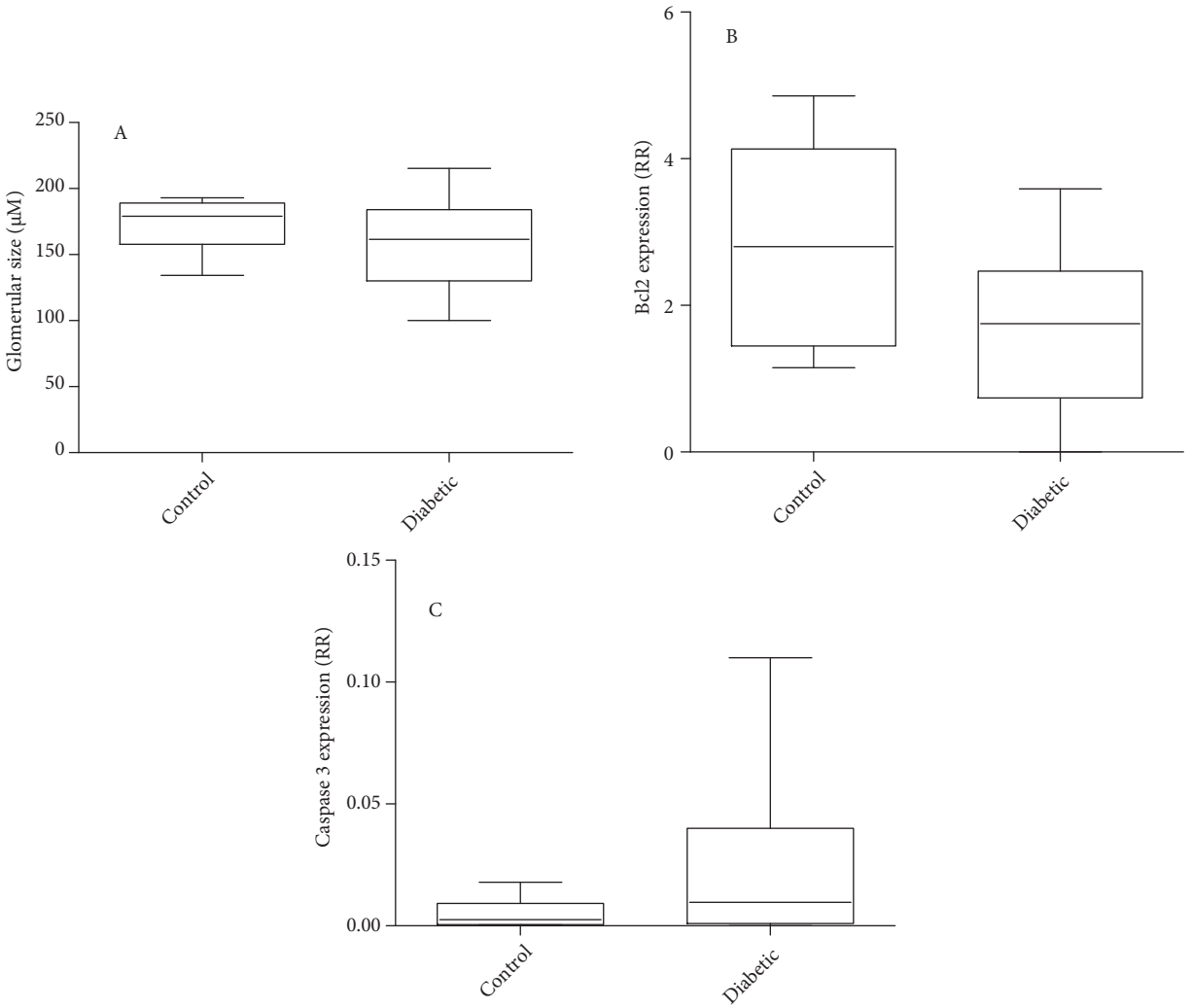

Figure 3. A) Glomerular diameter of the control and diabetes groups. Two sections of renal tissues per animal were examined histomorphometrically at ×20 magnification. Measurements of the glomerular diameter (μm) were performed on bilateral visceral epithelium of 50 glomeruli. B) Gene expression levels of Bcl-2. C) Gene expression levels of caspase-3 in the control and diabetes groups. Gene expression levels were normalized to G6PDH (RR = relative ratio).

Discussion

The aim of this study was to investigate the effects of diabetes on the renal tissues that mainly underlie the mechanism of apoptosis and the NO pathway. The STZ-induced hyperglycemic rat model used here simulates human noninsulin-dependent diabetes, and is also inexpensive and easily reproducible (14). NO is synthesized in 2 steps from the amino acid L-arginine (L-Arg) along with L-citrulline (15). This reaction is catalyzed by NOS (16). The expression of iNOS is stimulated by inflammatory cytokines, and, once activated, iNOS can produce up to 1000-fold more NO than eNOS (17). High glucose concentrations have been shown to increase cytokine-induced protein and gene expression levels of iNOS in rat renal tissues (18,19). It is possible that the iNOS expression level can be decreased by insulin, which represses the effects of hyperglycemia and inflammatory cytokines. Many experimental studies have been performed to elucidate the role and regulation of the NO system in vivo in regard to vascular reactivity, NOS expression, and modulation of hemodynamics, all of which are the areas in which the most change occurs during early DN (20). The role of iNOS during DN is not clearly understood. Experimental studies have demonstrated that iNOS is expressed

Table. Glomerular size and gene expression levels of Bcl-2 and caspase-3 in the control and diabetes groups.

	Group (n = 10)	Mean*	P**
Glomerular size (μm)	Control	176.45 ± 23.41	0.395
	Diabetes	183.91 ± 26.49	
Bcl-2 expression (RR)	Control	3.033 ± 0.440	0.030
	Diabetes	1.517 ± 0.438	
Caspase-3 expression (RR)	Control	0.016 ± 0.015	0.599
	Diabetes	0.025 ± 0.016	

Gene expression levels were normalized to G6PDH (RR = relative ratio).
*± standard deviation, **Student's t-test.

in various cell types and that it possibly contributes to increased NO generation, hyperfiltration, and diabetic glomerular changes (21,22). The endothelial expression of iNOS in structurally preserved diabetic glomeruli and peritubular capillaries indicates that it might be induced due to early endothelial alterations, and thereby contributes to increased endothelial NO production during DN. We investigated the expressions of iNOS and eNOS in the renal tissues of diabetic rats.

Our findings clearly strengthen the view that NO activity is stimulated in proximal renal tubule cells and renal medulla in diabetes, but does not change in the glomeruli or the Bowman capsule. These findings reveal that the length of the diabetic status is very important, and that the renal cortex is the most resistant area to diabetes-induced damage. In rodents, eNOS expression is usually weak in the first week after birth, but becomes stronger after weaning (23). In endothelial cells, hyperglycemia inhibits eNOS activity leading to reduced NO and increased ROS production (24). NOS inhibition is also involved in glomerular cell loss and apoptosis, and in the pathophysiological changes in severe nephrosclerosis and impaired renal dynamics (25). Furthermore, eNOS was detected throughout the rats' vasculature, including the glomerulus (6). Other studies claimed that the main stimuli for eNOS activation are probably shear stress and pulsatile stretch (26). Glomerular eNOS expression correlated with the degree of vascular injury as assessed by a vascular injury score (20). The increase of eNOS in diabetic glomeruli was likely to reflect the pool of enzyme not active in eNOS generation, but in superoxide production. An increased level of eNOS expression was found in microvessels and renal tissues of diabetic rats. This was in agreement with other studies that also used diabetic rats as their experimental disease model (16,27,28). Only one study with early DM rats detected no changes in renal eNOS protein expression (29). This result may be related to the time course of diabetes induction (28). We found decreased eNOS expression in the proximal renal tubule cells, glomerulus, and Bowman capsule in the diabetic group when compared with the healthy controls. This decrease might reflect how the endothelial cell group still behaves as if under normal physiological conditions although it is under the subacute effects of diabetes, and how the inhibition of eNOS due to hyperglycemia and the increase of eNOS triggered by shear stress and pulsatile stretch did not impair the balance in favor of one of these states. The effects of apoptosis become more profound in the course of this progression and the impairment of this balance will be unavoidable. After injury, mesangial cell death by apoptosis is known to be involved in the resolution of glomerular hyper-cellularity, which suggests that apoptosis is a homeostatic mechanism regulating glomerular cell population (30). Similar to mesangial cell death, we observed endothelial cell loss in our

study. There are many high glucose-induced cell death reports that describe mechanisms that overlap very well with what we observed. Judging from the aforementioned conditions, a model for high glucose-induced apoptosis can indeed be proposed. The initial cellular response to a high-glucose environment is the generation of various ROS forms. This early and critical event in high glucose-induced cell death has several important stages, which begin with the activation of key enzymes in the polyol pathway that are probably linked to glucose transporters at the cell membrane. The generation of reactive nitrogen species, in combination with ROS, rapidly induces apoptotic and necrotic cell death via mitochondria-dependent and mitochondria-independent pathways. It is obvious that high glucose levels affect several stages of apoptotic signaling, resulting in cell death by increasing oxidative and nitrosative stress, which in turn activate the proapoptotic Bcl-2 protein family members and initiate the caspase cascade. It is also clear that apoptosis leads to the pathology of mesangial cell loss associated with progressive glomerulosclerosis (31). In agreement with all these studies, which were undertaken either in early- or late-stage diabetes, we also found a decrease in Bcl-2 gene expression. Besides this, we also found an increase of caspase-3 activation in proximal renal tubule cells by immunohistochemical analyses. Together, these results for increased caspase-3 and decreased Bcl-2 expression demonstrate that the degree of apoptotic activity is higher under diabetic conditions than normal conditions.

The current study shows that NOS activity and Bcl-2 and caspase-3 gene expressions in the renal tissue of STZ-induced diabetic rats are dysregulated since hyperglycemia inhibited eNOS activity, which correlated with glomerular cell loss due to apoptosis, and increased iNOS expression was associated with endothelial dysfunction.

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