Nuclear Factor-κB Activation in Diabetic Rat Kidney: Evidence for Involvement of P-Selectin in Diabetic Nephropathy

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IWAMOTO, M., MIZUIRI, S., ARITA, M. and HEMMI, H. Nuclear Factor-κB Activation in Diabetic Rat Kidney: Evidence for Involvement of P-Selectin in Diabetic Nephropathy. Tohoku J. Exp. Med., 2005, 206 (2), 163-171 — Activation of a transcription factor, nuclear factor-κB (NF-κB), is a key step in the pathogenesis of diabetic nephropathy. In this study, we investigated the role of P-selectin, a platelet-derived adhesion molecule, in diabetic nephropathy by examining the activation status of NF-κB in the renal cortex of streptozotocin (STZ)-treated rats. The STZ treatment induced pathogenetic parameters such as increased creatinine clearance, increased blood glucose and massive albuminuria in a time-dependent manner. Electrophoretic mobility shift assays (EMSA) with a specific probe, representing the P-selectin gene promoter, revealed the activation status of NF-κB in the STZ-treated rats, as judged by the time-dependent increase in the formation of the specific protein-DNA complexes. This increase was associated with the increased pathogenetic parameters. Supershift assays with specific antibodies revealed that p50, but not p52, p65, Rel B, or c-Rel, may be involved in the activation of NF-κB, though the component primarily responsible for the increase could not be determined. Western blot analysis confirmed an increase in P-selectin in STZ-treated rats. Notably, treatment with ammonium pyrolidinedithiocarbamate, an antioxidant and inhibitor of NF-κB, inhibited the activation of NF-κB in STZ-treated rats and decreased P-selectin in the renal cortical tissue. Our results indicate that expression of the P-selectin gene is induced through the activation of NF-κB and that P-selectin may be involved in the pathogenesis of diabetic nephropathy.

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Diabetic nephropathy (DN) is a serious complication of diabetes mellitus and is characterized by functional and structural abnormalities including albuminuria and extracellular matrix accumulation (Narita et al. 1999; Cooper 2001; Adamczak et al. 2002). Continuous hyperglycemia induces a wide variety of biochemical events such as expression of the angiotensinogen gene and oxidative stress, including the expression of inducible nitric oxide (NO) synthetase in renal cells (Wang et al. 1998; Zhang et al. 1999; Noh et al. 2002; Satoh et al. 2002). Inhibitors of angiotensin-con-
verting enzyme (ACE) and angiotensin II receptor blockers are thought to be involved in the development of DN (Bjorck et al. 1986; Whitty and Jackson 1988; Onozato et al. 2002). We previously reported the frequent occurrence of nephropathy in diabetic patients with genotype D of the ACE gene (Mizuiri et al. 1995, 1997) and that, in the kidney from the D genotype population, the number of transcripts of the ACE gene is higher than in I genotype (Mizuiri et al. 2001), suggesting that ACE activity contributes to the development of DN. The ACE product, angiotensin II, and its degradative product angiotensin III activate the transcription factor, nuclear factor-κB (NF-κB) (Cooper 2001; Guijarro and Egido 2001). NF-κB is activated by translocation from cytoplasm to nucleus and the formation of a heteromeric complex consisting of several components including p50, p52, p65, Rel B, and c-Rel (Border and Noble 1993).

Activation of NF-κB during hyperglycemia has been reported in many cell types, such as human peripheral blood mononuclear cells (Hofmann et al. 1998), porcine vascular smooth muscle cells (Yerneni et al. 1999), rat kidney cortex (Morrissey and Klahr 1997; Rangan et al. 1999) and human proximal tubular cells (Jones et al. 2001). Activation in nuclei of these cells is induced by second messengers, because it is unlikely that glucose itself could interact with NF-κB or its components. In this regard, angiotensins (Wang et al. 1998; Zhang et al. 1999), oxidative stress (Wolff 1993; Noh et al. 2002), and various cytokines such as interleukin (IL)-1 (Hasegawa et al. 1991), IL-6 (Suzuki et al. 1995), transforming growth factor-β (Border and Noble 1993; Cooper 2001), tumor necrosis factor (Hasegawa et al. 1991), and vascular endothelial growth factor (Grone et al. 1995), all of which are known to activate NF-κB (Guijarro and Egido 2001), can cause kidney damage. Many of these cytokines are produced by hematopoietic mononuclear cells including monocytes and macrophages, suggesting that infiltrating monocytes/macrophages in the kidney play a critical role in the development of chronic renal injury (Hofmann et al. 1999). Furthermore, the accumulation in the kidney of adhesion molecules such as vascular cell adhesion molecule-1 (Seron et al. 1991), intracellular adhesion molecule (ICAM)-1 (Sugimoto et al. 1997), and P-selectin (Hirata et al. 1998) may induce structural damage. P-selectin is expressed on activated endothelial cells and platelets (Geng et al. 1990) and regulates the initial interactions between leukocytes and the blood vessel wall as well as between activated platelets and leukocytes (Vandendries et al. 2004). In many tissues, the expression of the genes of these adhesion molecules including P-selectin is also activated by NF-κB (Guijarro and Egido 2001). Thus, activation of NF-κB leads to the expression of the adhesion molecules, which may directly or indirectly cause kidney injury.

In this study, we investigated the activation of NF-κB focusing on P-selectin expression in the renal cortex of streptozotocin (STZ)-treated rats, a widely utilizing animal model of diabetes mellitus (Portha et al. 1979, 1989; Coskun et al. 2004). Our results demonstrate that P-selectin may be involved in the onset and progression of DN through NF-κB activation.

MATERIALS AND METHODS

Animals and experimental protocols

Male Wistar rats weighing 220 to 250 g were obtained from Nihon SLC, Inc., (Tokyo). The rats were housed under controlled environmental conditions (12/12 hours dark/light cycle at 22°C) and had free access to standard rat chow and water. Diabetes was induced in one group of rats (n = 5 to 12) by intravenously injecting STZ (50 mg/kg body weight; Sigma Chemical Co., St. Louis, MO, USA), freshly prepared at a concentration of 20 mg/ml in saline, into the tail. Two days later, induction of diabetes was confirmed by measuring the blood glucose level. Untreated rats (n = 4 to 7) were housed under the same conditions as the STZ-treated rats and used as controls. On days 7, 60, 150, and 240 after the injection, rats were anesthetized and sacrificed by exsanguination. Both kidneys were removed, weighed, and processed for analysis. One day prior to sacrifice, the rats were placed in metabolic cages to determine 24 h urinary albumin and creatinine excretion. Venous blood was collected at the time of sacrifice to determine albumin and serum creatinine levels. Serum glucose was determined by an electro-enzymatic system (model
GA1160, Arkay Co., Kyoto). Urinary albumin and serum creatinine were determined by a double antibody radioimmunoassay (Keen and Chlouverakis 1963) with small modifications and an IatroLQ CRE kit (Mitsubishi Kagaku Iatron, Inc., Tokyo), respectively. In some experiments, freshly prepared ammonium pyrrolidinedithiocarbamate (PDTC) (Sigma Chemical Co.), an NF-κB inhibitor (Schreck et al. 1992), was administered at 100 mg/kg body weight to some of the 240-day-rats. These rats were sacrificed 4 hours after the administration of PDTC. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Toho University School of Medicine.

Preparation of nuclear extracts
Renal cortical tissue was carefully dissected from the medulla, immediately snap frozen in liquid nitrogen, and stored at −80°C until further processing. Nuclear protein extracts were prepared according to the methods of Rangan et al. (1999), with some modifications. Cortical tissue (100 mg) was homogenized with 20 even strokes of a homogenizer (Wheaton, Millville, NY, USA) in 200 μl of ice-cold buffer A at 4°C. Buffer A was composed of 10 mM 4-(2-hydroxyethyl)piperidine-1-ethanesulfonic acid (HEPES) (pH 7.9), 10 mM KCl, 2 mM MgCl₂, 0.1 mM ethylenediaminetetraacetic acid (EDTA), and a cocktail of protease inhibitors (0.5 mM dithiothreitol, 1 μM phenylmethylsulfonyl fluoride, 0.05 μg leupeptin/ml, and 10 μg aprotinin). Then, 65 μl of nonidet-P 40 (2%) were added. The mixture was vortexed for one minute, incubated on ice for 10 minutes, and centrifuged at 13,000 × g for 5 minutes. The supernatant was carefully removed and the pellet was resuspended in 60 μl of buffer B, containing 50 mM HEPES, 10% (vol/vol) glycerol, 300 mM NaCl, 50 mM KCl, and the protease inhibitor cocktail. The mixture was vortexed for one minute at 15 minutes intervals. Thirty minutes later, the mixture was centrifuged at 13,000 × g for 10 minutes. The supernatant, which contained nuclear protein, was stored at −80°C. Protein concentrations were determined by the Bradford method (Bradford 1976) using a Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Hercules, CA, USA).

Electrophoretic mobility shift assay (EMSA)
A double-stranded oligonucleotide of P-selectin (TAAGGAGCCAGGGACTTTACTGCATGCATA) with 7 of 9 conformities in the NF-κB consensus core sequence (underlined) (Lenardo and Baltimore 1989) was chosen from a sequence of the promoter region of the human P-selectin gene (GenBank accession number L01574). A mutated P-selectin oligonucleotide (TAAGGAGCCAAAAACCTTATGCATGCATA) with low conformity (4/9, boldfaced) was also synthesized.

Fifteen pmols of double-stranded oligonucleotide was end-labeled with 150 μCi of (γ-32P) ATP (NEN Life Science Products, Inc., Boston, MA, USA) in a reaction mixture containing 20 units of T4 polynucleotide kinase (New England Biolabs, Inc., Beverly, MA, USA), 70 mM Tris-HCl, 10 mM MgCl₂, and 5 mM dithiothreitol. The binding reaction was run at 37°C for 60 minutes and was stopped by incubation at 75°C for 10 minutes. Radiolabeled oligonucleotide was purified using G-50 Sephadex spin columns (Roche Diagnostic, Mannheim, Germany). The binding reaction mixture contained 7.5 μg of nuclear extract, 2 μl of binding buffer (5 mM MgCl₂, 50 mM Tris-HCl, 250 mM NaCl, 20% glycerol, 2.5 mM EDTA, 2.5 mM dithiothreitol, and 0.25 mg/ml poly[2’-deoxyinosinic-2’-deoxyctydylid acid] [Sigma Chemical Co.],) and 1 μl of the 32P-labeled P-selectin probe (50,000 cpm, 0.5 ng/μl) in a total volume of 10 μl, and was incubated for 30 minutes at room temperature. The reaction was stopped by the addition of 1 μl of gel-loading buffer (250 mM Tris-HCl, pH 7.5, 0.2% bromophenol blue, and 40% glycerol). The mixture was subjected to electrophoretic separation performed on a 7% polyacrylamide gel/0.5 × Tris-borate-EDTA electrophoresis buffer consisting of 89 mM Tris base, 89 mM boric acid, and 2 mM EDTA, pH 8.0. The radioactivity of each band on the dried gel was directly measured with an Instant Imager (Packard Co., Tokyo) and the bands were visualized using a phosphoimager, BAS-2000 (Fuji Film Co., Tokyo).

Supershift assay
To determine the subunit composition of the NF-κB complexes, supershift assays were performed using specific concentrated rabbit antisera reactive to epitopes on rat p50, p52, p65, Rel-B, or c-Rel (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Rabbit IgG was used as an antibody control. Two micrograms of antibody were added 30 minutes after the addition of the labeled probe and incubated at room temperature for an additional 30 minutes. Electrophoresis with a 7% polyacrylamide gel was performed similar to EMSA.
Western blotting

Frozen cortical tissue (1 g) in 1 ml of ice-cold lysis buffer consisting of 2 × phosphate-buffered saline (136.9 NaCl, 2.7 KCl, 8.1 Na₂PO₄, and 1.5 KH₂PO₄, pH 7.3, [in mM]), 1% nonidet-P 40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 10 μg leupeptin/ml, and 1 mM pefabloc SC (E. Merck, Darmstadt, Germany) was passed through a syringe with a 18, 21, 23, or 25 G needle, and homogenized by Dounce homogenizer with a tight pestle. The homogenized solution was centrifuged at 10,000 × g, 4˚C for 20 minutes. The protein concentration of the supernatant was determined and the supernatant was stored at –80˚C prior to use. The supernatant (200 μg/lane) was applied to an SDS-7.5% polyacrylamide gel and electrophoresed. Proteins on the gel were electroblotted to a polyvinylidene difluoride (PVDF) membrane (Hybond-P; Amersham Pharmacia Biotech, Inc., Piscataway, NJ, USA) and the membrane was cut between the bands of 62 and 83 kDa of the molecular weight marker (prestained protein marker, broad range, New England Biolabs, Beverly, MA, USA). The upper part of the membrane was soaked with mouse monoclonal antibody against P-selectin (CTB201, Santa Cruz Biotechnology) at a concentration of 2 μg/ml. The lower part was soaked with mouse monoclonal antibody against actin (Sigma Chemical Co.) diluted 1:500. Specific bands were visualized using a ProtoBlot II AP system (Promega Co., Madison, WI, USA).

Statistical analysis

Data were expressed as the mean ± s.d. Differences between parameters were analyzed using the Mann Whitney’s U-test. P values less than 0.05 were considered statistically significant.

RESULTS

NF-κB Activation and development of DN

The relationship between NF-κB activation and development of DN was studied in STZ-treated rats. Characteristic features of DN such as an increase in blood glucose concentration, reduced creatinine clearance, and albuminuria were observed in rats 7 days or later after the treatment with STZ, while spontaneous increase in albuminuria was seen in the untreated rats with lesser extent (Table 1). The increase in the blood glucose in some of the untreated rats observed may occur by ad libitum feeding. Activation of NF-κB was measured by EMSA with a probe derived from a promoter region of the human P-selectin gene promoter and nuclear extracts of renal cortex isolated from STZ-treated rats. Firstly, the specificity of the probe was determined with nuclear extracts isolated at day 60. Two major shifted bands (bands 1 and 2) were observed (Fig. 1, lane 2). Neither of the bands specific to NF-κB was

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<th>Duration days</th>
<th>STZ treatment</th>
<th>Number of rats</th>
<th>Body weight (g)</th>
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<th>Creatinine clearance (ml/min/kg)</th>
<th>Albuminuria (μg/day)</th>
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Values are expressed as means ± s.d.  
* p < 0.05 and **p < 0.01 when compared to the corresponding untreated group.
affected by the addition of an excess amount (100-times) of the cold mutated oligonucleotide (Fig. 1, lane 4), though the bands were clearly diminished by the same excess amount of the wild-type oligonucleotide (Fig. 1, lane 3), indicating that the P-selectin oligonucleotide binds specifically. We then examined the increase over time of the two bands with STZ treatment. An increase of band 2 was observed at day 7 and later in STZ-treated rats; the intensity of the bands from untreated rats also increased, but to a lesser extent (Fig. 2A). The radioactivity of band 2 of STZ-treated rats was significantly higher than untreated rats after day 7 to day 150 (Fig. 2B). The intensity and radioactivity of band 1 remained unchanged in both groups (Fig. 2). The increase in band 2 in untreated and STZ-treated rats paralleled the increase in albuminuria (Table 1).
Increased component of NF-κB by STZ treatment

NF-κB is known to consist of heteromers such as p50, p52, p65, Rel B, and c-Rel (Border and Noble 1993). We next examined the effect of STZ treatment on each of these component. Supershift assays with each specific antibody showed a major supershifted band accompanied by the disappearance of band 1 but not band 2 only with anti-p50 antibody (Fig. 3). The radioactivity of band 2 in the presence of anti-p50 antibody decreased 15 to 20% compared to that in the absence of the antibody (data not shown), indicating that p50 is one of the components of both bands 1 and 2. The use of antibodies against p52, p65, Rel B, and c-Rel as well as rabbit IgG essentially had no effect (Fig. 3, lanes 4-8). No signifi-

Fig. 3. Determination of increased NF-κB components in DN-rats by super gel shift assay. Nuclear extracts isolated from a DN-rat at day 60 and a radioactive probe for P-selectin were incubated in the absence (lane 2) or in the presence of antibody against p50 (lane 3), p52 (lane 4), p65 (lane 5), Rel B (lane 6) or c-Rel (lane 7), or normal rabbit IgG (lane 8). The reaction mixture was separated by PAGE and visualized by a phosphoimager. No nuclear extract was used as a negative control (lane 1). Migration of free probe is shown by F.

Fig. 4. Increase in P-selectin in renal cortex of STZ-treated rats by effect of PDTC. A: Western blot analysis of P-selectin. Cell lysates were prepared from renal cortex of a untreated rat at day 7 (lane 1), and a rat treated with STZ at days 7 (lane 2) and 240 (lane 3), and day 240 with PDTC administered 4 h before sacrifice (lane 4), then electrophoresed on an SDS-7.5% polyacrylamide gel. The gel was electroblotted onto a PVDF membrane and the membrane was treated with anti P-selectin mouse antibody. The band was visualized with alkaline phosphatase-conjugated anti-mouse IgG. B: EMSA analysis. Nuclear extracts prepared from the same rats described in A. EMSA was performed with radioactive P-selectin oligonucleotide as a probe. Migration of free probe is shown by F.
significant decrease in the radioactivity of bands 1 and 2 was detected when these antibodies were used (data not shown). The results indicate that p50 is involved in the activation of NF-κB. However, the major component of band 2 could not be determined.

Increased expression of P-selectin and inhibition by PDTC

Since expression of the P-selectin gene is involved in the development of DN through activation of NF-κB, we used Western blot analysis to determine whether an increase in P-selectin protein occurs in the kidney cortex of STZ-treated and untreated rats. A 122-kDa band corresponding to P-selectin was detected in cell lysates of STZ-treated rats at day 240 (Fig. 4A, lane 3), but not those from day 7 (lane 2). Further, PDTC, an NF-κB inhibitor (Schreck et al. 1992) known to suppress nephropathy-related symptoms (Rangan et al. 1999; 2001), significantly decreased both STZ-induced NF-κB activation (Fig. 4B, lane 4) and the intensity of the P-selectin band (Fig. 4A, lane 4). These results strongly suggest that P-selectin is involved in the pathogenesis and/or progression of DN.

DISCUSSION

The present study has shown the involvement of P-selectin in the development of DN through NF-κB activation in the renal cortex of STZ-treated rats, an animal model of DN (Portha et al. 1979, 1989; Coskun et al. 2004). This finding is consistent with previous observations of increased P-selectin expression in kidneys of patients with DN (Hirata et al. 1998) and of upregulated human P-selectin gene expression due to NF-κB activation (Pan and McEver 1995; Weyrich et al. 1995). To our knowledge, this is the first in vivo study that suggests a correlation between DN and NF-κB activation. Our results also showed that PDTC, an inhibitor of NF-κB, reduced P-selectin expression at the protein level probably through the inhibition of NF-κB activation in STZ-induced DN rats. This suppression suggests the role of P-selectin in the development of DN. Thus NF-κB inhibitors might be useful for improving DN status.

A positive correlation between biochemical parameters of DN and NF-κB activation was observed with the P-selectin oligonucleotides used as a probe. The consensus sequence was from the human gene, so there may be a species-specific difference between the corresponding human and rat genes. During the preparation of this manuscript, the rat genome project using Rattus norvegicus was completed (Rat Genome Sequencing Project Consortium 2004). We searched for the NF-κB consensus core sequence to a point 4,000 bp upstream of the translation starting site of the P-selectin gene in the database. The core sequence was found at -1,459 to -1,451 with 7/9 conformity. The conformity to the probe used was 14/30 with 7/9 in the core sequence. The homology may be sufficient to detect NF-κB activation, though further studies are necessary to investigate the correlation with a species-specific probe. A mutated probe with a disrupted NF-κB consensus core sequence had no effect on band intensity in the competitive EMSA, indicating that the activation was seen in NF-κB binding and was sequence-specific. Furthermore, the activation detected with the P-selectin probe may involve NF-κB-activating genes other than the P-selectin gene.

The accumulation of P-selectin protein in the kidneys of DN rats was confirmed by Western blot analysis. At day 7 after treatment, when significant albuminuria was evident, no increase in band intensity was observed; at day 240, the increase was significant. Such increases indicate that P-selectin protein accumulates the kidney of DN rats. This observation supports the finding of increased P-selectin expression in kidneys of patients with DN (Hirata et al. 1998). We also demonstrated that the accumulation of P-selectin could be inhibited by administration of PDTC at 4 hours prior to the sacrifice, indicating that the accumulation in the renal cortex is caused by NF-κB activation. Indeed, under the same conditions, inactivation of NF-κB was observed. Further studies are needed to determine the relationship between renal failure and accumulation of P-selectin in kidney.
Using the P-selectin probe, we found two major bands in untreated and STZ-treated rats by EMSA. An approximately two-fold increase in the intensity of band 2, but not band 1, was noted in STZ-treated rats compared with untreated rats. Supershift assays did not reveal the major component(s) of band 2; 15-20% was p50, which was also a major component of band 1. Increases of p50 and p65 have been reported in human mesangial cells (Guijarro and Egidio 2001) and in kidney of doxorubicin-treated rats (Rangan et al. 2001). In our supershift assays, anti-p52, anti-p65, and anti-Rel B antibodies seemed to possess some suppressive effect on both bands, though this was not significant when the changes in radioactivity were measured quantitatively (unpublished results). These results suggest that p52, p65, and Rel B participate at least in part in NF-κB activation in DN rats. It should be noted, however, that the aged Wistar rats showed kidney damage due to nephrosclerosis, and that gradual increases in the blood glucose and albuminuria were seen in untreated rats in the present study after day 60 of feeding.

In conclusion, P-selectin is involved in the development of DN through NF-κB activation. A high glucose concentration is known to activate NF-κB in vitro (Morrisey and Klahr 1997; Hofmann et al. 1998; Rangan et al. 1999; Yemeni et al. 1999; Jones et al. 2001). To our knowledge, this is first in vivo study to suggest a correlation between DN and NF-κB activation. P-selectin, an adhesion molecule expressed on the endothelium and released from platelets, acts as a mediator for blood mononuclear cells rolling on the endothelium (Bonfanti et al. 1989; Larsen et al. 1989). A rat P-selectin probe available since the completion of the rat genome project (Rat Genome Sequencing Project Consortium 2004), should provide useful in confirming the role of P-selectin and other NF-κB-associated proteins in the pathogenesis and progression of DN in STZ-treated rats.

Aknowledgments

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References


expression in rat diabetic nephropathy.


