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Peripheral blood mononuclear cells isolated from patients with diabetic nephropathy show increased activation of the oxidative-stress sensitive transcription factor NF-κB

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Summary Increased oxidative stress and subsequent activation of the transcription factor NF-κB has been linked to the development of late diabetic complications. To determine whether oxidative stress dependent NF-κB activation is evident in patients with diabetic nephropathy we used an Electrophoretic Mobility Shift Assay based semiquantitative detection system which enabled us to determine NF-κB activation in ex vivo isolated peripheral blood mononuclear cells. We examined 33 patients with diabetes mellitus (Type I and Type II). Patients with diabetic nephropathy showed higher NF-κB binding activity in Electrophoretic Mobility Shift Assays and stronger immunohistological staining for activated NF-κBp65 than patients without renal complications. NF-κB binding activity correlated with the degree of albuminuria ($r = 0.316$) and with thrombomodulin plasma concentrations ($r = 0.33$), indicative for albuminuria associated endothelial dysfunction. In a 3 day intervention study in which 600 mg of the antioxidant thioctic acid (α-lipoic acid) per day were given to nine patients with diabetic nephropathy oxidative stress in plasma samples was decreased by 48 % and NF-κB binding activity in ex vivo isolated peripheral blood mononuclear cells by 38 %.

In conclusion, activation of the transcription factor NF-κB in ex vivo isolated peripheral blood mononuclear cells of patients with diabetes mellitus correlates with the degree of diabetic nephropathy. NF-κB activation is at least in part dependent on oxidative stress since thioctic acid (α-lipoic acid) reduced NF-κB binding activity. [Diabetologia (1999) 42: 222–232]

Keywords Oxidative stress, nephropathy, transcription factor NF-κB, thrombomodulin, thioctic acid.

Increased oxidative stress generated by hyperglycaemia, hyperlipidaemia or the presence of advanced glycation endproducts (AGEs) or both is regarded as an important mediator of late diabetic complications [1]. A clear relation between the quality of glycaemic control and the presence of free radicals has been shown thereby relating the appearance of oxidative stress to the underlying metabolic disorder rather than to the complication itself [2]. The presence of oxygen free radicals and the simultaneous decline of antioxidative defence mechanisms observed in diabetic patients could therefore promote the development of late diabetic complications [1, 3–5].

Oxidative stress has been proposed as a major cause of diabetic nephropathy [6–9]. Animal models implicate that oxidative stress accompanying the early onset of diabetes increases the kidney's susceptibility to develop diabetic nephropathy [10]. One possible mechanism for this is the activation of the oxidative stress sensitive transcription factor NF-κB [6, 11–13]. Translocation of activated NF-κB into the nucleus results in gene expression of NF-κB controlled

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Abbreviations: AGEs, Advanced glycation endproducts; BMI, body mass index; EMSA, electrophoretic mobility shift assay; 4-HNE, 4-hydroxynonenal; MDA, malonaldehyde; NF-κB, nuclear factor-kappa B; PBMC, peripheral blood mononuclear cells; PBS, phosphate buffered saline; rNF-κBp65, recombinant nuclear factor-kappa B subunit p65.

Table 1. Characterisation of the 33 patients included in the cross-sectional study. Values represent the mean ± SD obtained from 33 patients with diabetes mellitus, divided into two groups based on the urine albumin concentration as sign for diabetic nephropathy

	without diabetic nephropathy (Urine albumin concentration < 20 mg/l) $n = 12$	with diabetic nephropathy (Urine albumin concentration > 300 mg/l) $n = 21$	p-Value
Sex (m/f)	6/6	11/10	0.899
Age (years)	41.4 ± 12.2	53.3 ± 13.0	0.014
Duration of diabetes mellitus (years)	16.3 ± 8.5	12.7 ± 11.7	0.358
HbA_{1c} (%)	7.2 ± 1.09	7.4 ± 0.994	0.67
Urine albumin concentration (mg/l)	7.25 ± 4.63	106.5 ± 62.2	0.00004
BMI (kg/m^2)	22.9 ± 2.7	26.1 ± 2.62	0.0019
Cholesterol (mg/dl)	183.8 ± 39.1	223.6 ± 57.1	0.041
Systolic blood pressure (mm Hg)	134.1 ± 22.13	126.5 ± 1499	0.243
Diastolic blood pressure (mm Hg)	78.3 ± 6.05	75.2 ± 8.08	0.262
Antihypertensive drugs	2 (12)	17 (21)	0.009
Prevalence of retinopathy	4 (12)	7 (21)	0.792
Prevalence of neuropathy	3 (12)	10 (21)	0.212
rNF-κBp65 equivalents (μg/10 μg nuclear extract of PBMC)	6.6 ± 4.2	13.7 ± 9.4	0.0004
Thrombomodulin (ng/ml)	19.5 ± 10.3	34.0 ± 17.8	0.019

target genes as leucocyte adhesion molecules, cytokines, renal epithelial-inducible nitric oxide synthase (eNOS), monocyte attractant protein-1 (MCP-1), endothelin-1 and tissue factor [12, 14–20]. Inducible NF-κB activation as response to hypoxia [21], cytokines [22, 23], angiotensin-II (Morcos and Nawroth, unpublished) or AGE-proteins [16, 24, 25] has been shown in glomerular mesangial cells [11, 20, 22, 23], tubular cells ([17], Morcos and Nawroth, unpublished), renal epithelial cells [19], the endothelium [12, 16, 24, 25] and monocytes/macrophages [26, 27]. In addition, in animal studies higher renal expression of the NF-κB system in diabetic animals compared with healthy control animals has been observed [28].

Poor glycaemic control has been shown to induce activation of NF-κB in ex vivo isolated peripheral blood mononuclear cells (PBMC) of patients with Type I (insulin-dependent) diabetes mellitus [29]. To ascertain whether not only hyperglycaemia but also albuminuria can trigger a general increase in NF-κB activation in PBMC of patients with diabetes mellitus, we investigated 33 HbA_{1c}-matched patients with good glycaemic control by using a semiquantitative Electrophoretic Mobility Shift Assay (EMSA) based detection system [29]. In patients with diabetic nephropathy selected at random we did a 3 day intervention study to find out whether the observed activation of NF-κB could be lowered by daily treatment with the antioxidant thioctic acid (α-lipoic acid), which has been shown to be a potent inhibitor of oxidative stress of NF-κB activation in vitro and in vivo [16, 29–33].

Subjects and methods

Patients. From our outpatient clinic, 33 patients matched for HbA_{1c} (7.4 % ± 0.9) with diabetes mellitus (17 male, 16 female; age 49.9 ± 13.8 years; duration of diabetes 14.0 ± 10.7 years) were selected for our cross-sectional study. All patients underwent a full medical history including age, duration of diabetes, body mass index, eating habits, smoking habits, blood pressure, total cholesterol, urinary albumin concentration and a full examination to screen for diabetic complications (Table 1). Diagnosis of diabetic nephropathy was based on urinary albumin concentrations in the morning spot urine. Albuminuria was defined by urinary albumin concentrations higher than 21 mg/l. The clinical diagnosis of diabetic retinopathy was based on the examination of the ocular fundus after dilatation of the pupils by experienced ophthamologists. Diagnosis of diabetic neuropathy was based on clinical assessment and measurement of the vibration perception threshold. Diabetic macroangiopathic complications were diagnosed according to clinical finding, doppler sonography and angiopathy. Of the patients with nephropathy 81 % were on antihypertensive drugs; the patients that did not receive antihypertensive drugs entered the study when nephropathy was diagnosed. Patients treated with ACE-inhibitors and angiotension II-antagonists were excluded because angiotensin II modulates NF-κB activation (Morcos and Nawroth, unpublished). Patients taking vitamins or glucocorticoids were excluded from the study.

We selected at random nine patients matched for HbA_{1c} (5 male, 4 female; age 57.4 ± 7.2 years; duration of diabetes 12.3 ± 9.06; Table 2) with diabetic nephropathy and gave them 600 mg of the antioxidant thioctic acid (α-lipoic acid) per day orally for 3 days. All patients included in this intervention study underwent a full medical history as described above and were taking antihypertensive drugs (Table 2) except ACE-inhibitors, AT-II antagonists, vitamins, glucocorticoids or antioxidants (aspirin was permissible). The study was approved by the ethics committee of the Department of Medicine, University of Heidelberg and informed consent had been obtained from all patients.

Table 2. Characterisation of the 9 patients included in a 3 day intervention study with thioctic acid (α-lipoic acid). Nine at random selected patients with diabetic nephropathy were treated with 600 mg thioctic acid (α-lipoic acid) per day orally for 3 days. NF-κB binding activity and enaldehyde concentrations (as measured for oxidative stress) were determined before and after intervention. Values represent the mean ± SD

	before treatment with thioctic acid	after 3 days treatment with thioctic acid	*p*-value
Sex (m/f)	5/4		
Age (years)	57.4 ± 7.2		
Duration of diabetes mellitus (years)	12.3 ± 9.09		
HbA_{1c} (%)	8.08 ± 0.66		
Urine albumin concentration (mg/l)	107.4 ± 69.1		
BMI (kg/m^2)	26.2 ± 2.77		
Cholesterol (mg/dl)	215.33 ± 36.55		
Systolic blood pressure (mm Hg)	136.1 ± 13.64		
Diastolic blood pressure (mm Hg)	79.4 ± 10.4		
Antihypertensive drugs	9 (9)		
Prevalence of retinopathy	5 (9)		
Prevalence of neuropathy	5 (9)		
rNF-κBp65 equivalents (μg/10 μg nuclear extract of PBMC)	16.01 ± 7.31	9.00 ± 5.14	0.031
Enaldehyde concentration (μM) as measured for oxidative stress	5.01 ± 1.5	2.71 ± 0.96	0.005

Clinical laboratory tests. Urine albumin concentration was determined in three overnight urine collections and analyzed by the nephelometric method (BNA, Behring-Werke, Marburg, Germany). HbA_{1c} concentrations were measured by HPLC (Diamat, Biorad, Munich, Germany; normal range 4.5–6.1 %). Thrombomodulin was determined using a two-site enzyme-linked immunoabsorbent assay (Asserachrom-Thrombomodulin-ELISA, Diagnostica Stago, Asniéres, France) according to the manufacturer's instructions as described previously [34].

Colorimetric assay for lipid peroxidation. As markers for lipid peroxidation malonaldehyde (MDA) and 4-hydroxy-3(E)-nonenal (4-HNE) were determined as described previously [29] using the LPO-586 Kit (WAK-Chemie, Bad Soden, Germany) according to the manufacturer's instruction. Blood was collected into syringes containing 3.8 % sodium citrate (9:1; vol/vol) and centrifuged before 200 μl of plasma supernatant were incubated for 40 min at 45 °C with the chromogenic substrate R1, which reacts with MDA and 4-HNE to form stable chromophores with an absorbance maximum at about 586 nm. At the end of incubation samples were centrifuged at $10\,000 \times g$ for 10 min, the clear supernatant was used for measurement of absorbance at 586 nm and compared with standard curves for MDA and 4-HNE to give the enaldehyde concentrations (μM) of the plasma samples.

Preparation of blood smears and immunhistological detection of activated NF-κBp65. Whole blood was drawn into syringes containing 3.8 % sodium citrate (9:1; vol/vol) supplemented with 2 mmol/l of the antioxidant thioctic acid to avoid method-dependent activation of NF-κB. Blood smears were done immediately and fixed in 4 % paraformaldehyde by standard methods. Immunohistochemistry for cell typing was carried out using CD-3, CD-14 and CD-19 mouse monoclonal antibodies (Pharmingen, Hamburg, Germany). Staining for NF-κBp65 was done using a monoclonal antibody (Boehringer Mannheim, Mannheim, Germany), which exclusively recognizes the activated form of NF-κB [29, 35]. Sections were washed in phosphate buffered saline (PBS) pH 7.4, for 5 min before endogenous peroxidase activity was inhibited by treatment with 0.3 % hydrogen peroxide, dissolved in methanol, for 30 min at room temperature. Blocking was done with 0.6 % normal horse serum for 20 min at room temperature before monoclonal mouse antibodies (conc.: 0.01 μg/μl; 60 min; room temperature) were used as primary antibodies. Sections were washed 3×10 min in PBS, pH 7.4, and then blocking with 0.6 % normal horse serum was repeated for an additional 20 min. Signal detection was carried out with the Vectastain ABC kit (Vector Laboratories Inc., Burlingham, Calif., USA) according to the manufacturer's instructions. Peroxidase activity was visualized with DAB substrate (Vector Laboratories Inc.), after which the sections were counterstained with Hematoxylin (Vector Laboratories Inc.). Control samples for immunostaining were included in all stainings and the primary antibody and its replacement by PBS and matching concentrations of normal horse serum were omitted (data not shown). To visualize the experiments, PBMC were isolated before seeding and fixation on coverslips and stained as described above to ascertain the results obtained in crude blood smears.

Preparation of peripheral blood mononuclear cells (PBMC). Immediately after venipuncture PBMC were separated as described previously [26, 29]. We carefully loaded 10 ml whole blood, anticoagulated with 3.8 % sodium citrate (9:1; vol/vol) onto Ficoll Paque Plus gradient (Pharmacia, Freiburg, Germany) and centrifuged for 30 min at $500 \times g$ (without brakes) at room temperature [35, 36]. The PBMC containing band was aspirated and the cells were washed three times in PBS, pH 7.4, analysed microscopically and counted independently by two investigators. The cell number was adjusted to 1×10^6 PBMC/ml. For some experiments PBMC were fractionated by adherence to plasma-coated tissue culture dishes to separate lymphocytes and monocytes as described previously [38]. Where indicated, neutrophil granulocytes were isolated by Polymorphprep (Immuno GmbH, Heidelberg, Germany) as described [38].

Preparation of nuclear proteins. Nuclear extracts were prepared as described before [16, 26, 29]. In brief, 1×10^6 PBMC were lysed in 400 μl icecold buffer A (10 mmol/l HEPES-KOH, pH 7.9, at 4 °C, 1.5 mmol/l $MgCl_2$, 10 mmol/l KCl, 0.5 mmol/l DTT, 0.2 mmol/l PMSF), incubated on ice and centrifuged for 30 s at 15 000 rpm in a table centrifuge. The supernatant was discarded and the nuclear pellet was resuspended in 100 μl icecold buffer B (20 mmol/l HEPES-KOH pH 7.9 at 4 °C, 25 % glycerol, 1.5 mmol/l $MgCl_2$, 420 mmol/l NaCl, 0.2 mmol/l EDTA, 0.5 mmol/l DTT, 0.2 mmol/l PMSF), incu-

bated on ice for 20 min and centrifuged for 2 min as above. The supernatant containing nuclear proteins was immediately quickfrozen at –80 °C. Protein concentration was determined according to Bradford using a BCA assay (Pierce, Ill., USA) [40].

Electrophoretic Mobility Shift Assay (EMSA). For EMSA 10 μg of nuclear proteins were used. Binding of NF-κB to 1 ng of 5′-radiolabelled NF-κB consensus oligonucleotides (5′-AGT TGA GGG GAC TTT CCC AGG C-3′; approximately 50 000 cpm [Cerénkov]) was done for 20 min at room temperature in 10 mmol/l HEPES, pH 7.5, 0.5 mmol/l EDTA, 100 mmol/l KCl, 2 mmol/l DTT, 2 % glycerol, 4 % Ficoll, 0.25 % NP-40, 1 mg/ml bovine serum albumin (BSA) and 0.1 μg/μl poly d/dC by Pahl's method [40] as described previously [16, 26, 29]. Protein-DNA complexes were separated from the unbound DNA probe by electrophoresis through 5 % native polyacrylamide gels containing 2.5 % glycerol and 0.5 × Tris-Borat-EDTA (TBE) [16, 29, 41]). Gels were dried under vacuum and exposed for 48 to 64 h to Amersham Hyperfilms (Amersham, Braunschweig, Germany) at –80 °C with intensifying screens. Specificity of binding was ascertained by competition with a 160-fold molar excess of unlabelled consensus oligonucleotides and by characterisation using specific antibodies for the NF-κB subunits p65, p50, p52, cRel and relB (all obtained from Santa Cruz, Heidelberg, Germany) as described [26, 41, 42].

Synthesis of recombinant protein. Recombinant NF-κBp65 (rNF-κBp65) was synthesised according to Melton and Krieg's method [43] as described previously [29, 41] using the T7-promoter of the plasmid Rc-CMV-p65 (P. Baeuerle, Tularik, San Francisco, Calif., USA).

Semiquantitative determination of NF-κBp65 binding activity. To quantitate the NF-κB signal obtained from isolated PBMC, rNF-κBp65 was included in each EMSA on each gel. 2.5, 5 and 7.5 μg rNF-κBp65 containing lysate were used, the resulting NF-κB signals were quantitated by densitometry (Biorad) and used to establish a standard curve for each gel [29]. The densitometric value for the NF-κB signal of a given patient was converted into rNF-κBp65 equivalents by using the internal standard curve for rNF-κBp65 [29].

Statistical analysis. Data are expressed as the arithmetic mean ± standard deviation (SD). The significance of differences was determined using one-way analysis of variance (ANOVA). Correlation between variables were tested using Pearson's correlation analysis. Differences were deemed to be significant when p was less than 0.08.

Results

NF-κB binding activity in ex vivo isolated PBMC of 33 patients with diabetes mellitus matched for HbA_{1c} and good glycaemic control (Table 1) and ten control subjects without diabetes mellitus was studied using an EMSA-based semiquantitative detection system [29]. Nuclear extracts from PBMC were incubated with radiolabelled oligonucleotides spanning an NF-κB consensus sequence before being subjected to nondenaturing gel electrophoresis. In addition, binding reactions with three different concentrations

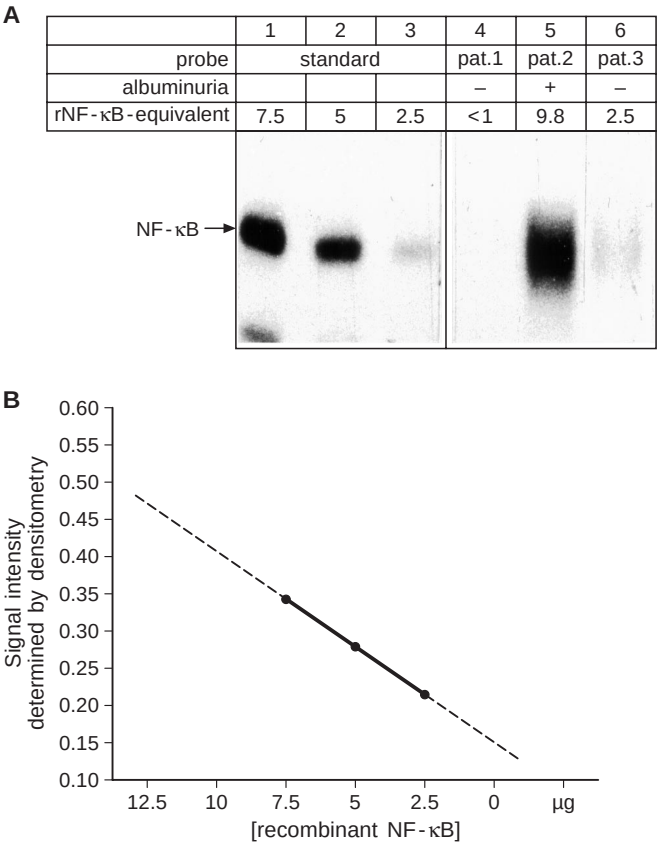


Fig. 1 A, B. The Electrophoretic Mobility Shift Assay (EMSA) based semiquantitative assay for the determination of NF-κB binding activity in ex vivo isolated peripheral blood mononuclear cells (PBMC): **A** EMSA-gel showing the internal standard with 7.5 μg, 5 μg and 2.5 μg recombinant NF-κBp65 (rNF-κBp65) (lane 1–3) and nuclear extracts derived from three different patients with diabetes mellitus (lane 4–6).The presence or absence of albuminuria is indicated by + and –. The densitometric intensity determined for each NF-κB signal is indicated below. **B** Example of a standard curve obtained for recombinant NF-κBp65

of rNF-κBp65 programmed rabbit reticulocyte lysate (7.5, 5, 2.5 μg) were separated on each gel and served as an internal standard (Fig. 1 a, lane 1–3) [29]. Densitometric evaluation of the signals obtained for the rNF-κBp65 standards allowed the construction of reproducible standard curves with an intrassay variability of 9 % (Fig. 1 b). The intensity of NF-κB binding activity in ex vivo isolated PBMC of a given probe (Fig. 1 a, lane 4–6) was determined by densitometry and converted into rNF-κBp65 equivalents, thereby providing a semiquantitative assay for the determination of NF-κB binding activity in a cross-sectional study.

When NF-κB binding activity in ex vivo isolated PBMC of control subjects without diabetes mellitus was compared with NF-κB binding activity in PBMC of patients with diabetes mellitus and good glycaemic control, a slight but not significant increase in NF-κB binding activity was observed (Fig. 2a; $p = 0.259$).

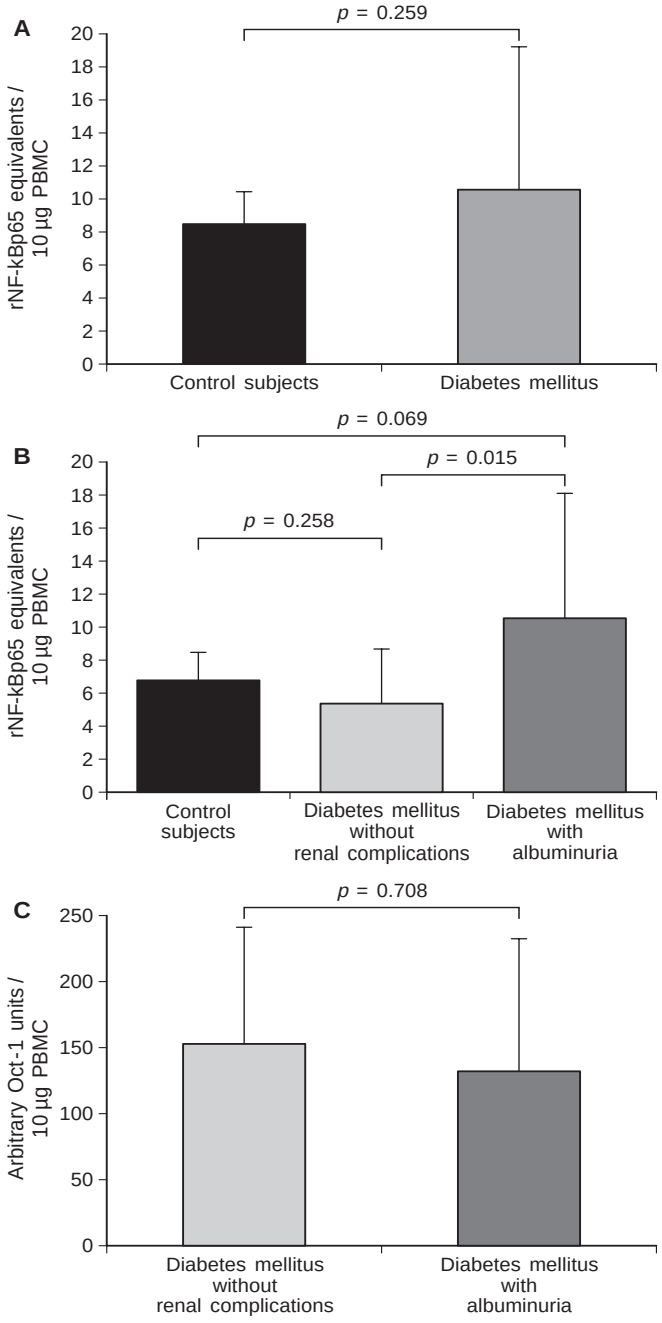


Fig. 2 A–C. NF-κB binding activity in ex vivo isolated PBMC is increased in patients with albuminuria: 10 µg nuclear extract of ex vivo isolated PMBC from 10 healthy control subjects and 33 patients with diabetes mellitus were assayed for NF-κB binding activity in EMSA. The signal was expressed as recombinant NF-κBp65(rNF-κBp65)-equivalent by using the internal standard curve for recombinant NF-κBp65 (**A**, **B**). To control for the quality of the nuclear extract, additional binding reactions were done with the ubiquitious transcription factor Oct-1. Densitometric evaluation of Oct-1 binding is expressed as arbitrary Oct-1 units (**c**). **A** NF-κB binding activity in PBMC of healthy control subjects and diabetic patients matched for HbA_{1c} with good glycaemic control. **B** NF-κB binding activity in PBMC of healthy control subjects, diabetic patients matched for HbA_{1c} without renal complications and patients matched for HbA_{1c} with albuminuria. **C** Oct-1 binding activity in PBMC of diabetic patients matched for HbA_{1c} without renal complications and patients matched for HbA_{1c} with albuminuria

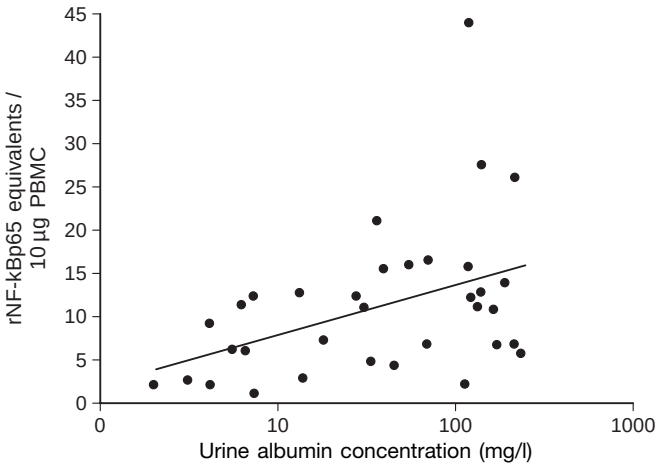


Fig. 3. NF-κB binding activity in PBMC correlates with albuminuria: 10 µg nuclear extract of PMBC from 33 patients with diabetes mellitus were assayed for NF-κB binding activity in EMSA. The signal was expressed as recombinant NF-κBp65(rNF-κBp65)-equivalent by using the internal standard curve for recombinant NF-κBp65 and plotted against urine albumin concentrations (mg/24 h) ($r = 0.316$; $p = 0.011$)

When all patients with diabetes were divided into two groups based on urine albumin concentration as a sign for diabetic nephropathy (Table 1), no difference was observed between healthy control subjects and diabetic patients matched for HbA_{1c} without renal complications (Fig. 2 b, $p = 0.258$), whereas significantly increased NF-κB activation was found in PBMC of diabetic patients with albuminuria (Fig. 2 b, $p = 0.069$ vs control subjects; $p = 0.015$ vs patients without renal complications). Since nuclear extracts of PBMC derived from patients without renal complications and from patients with albuminuria showed a constant activity for the transcription factor Oct-1 (Fig. 2c; $p = 0.708$), it could be almost excluded, that the observed changes in NF-κB binding activity were related to methodological variances. The data, therefore, indicate that albuminuria is associated with increased NF-κB activation in ex vivo isolated PBMC, even in the absence of hyperglycaemia. The extent of mononuclear NF-κB binding activity in ex vivo isolated PBMC significantly correlated with the severity of albuminuria (Fig. 3; Pearson's coefficient $r = 0.316$, $p = 0.011$). The correlation did not significantly differ in patients with nephropathy alone and patients with nephropathy in the presence of retinopathy or neuropathy or both (data not shown). No correlation was observed with age, duration of disease, BMI, cholesterol and blood pressure (Table 3). In all samples studied, proteins binding to the NF-κB consensus motif were characterised as NF-κB(p50/p65) by supershifting and competition experiments (data not shown).

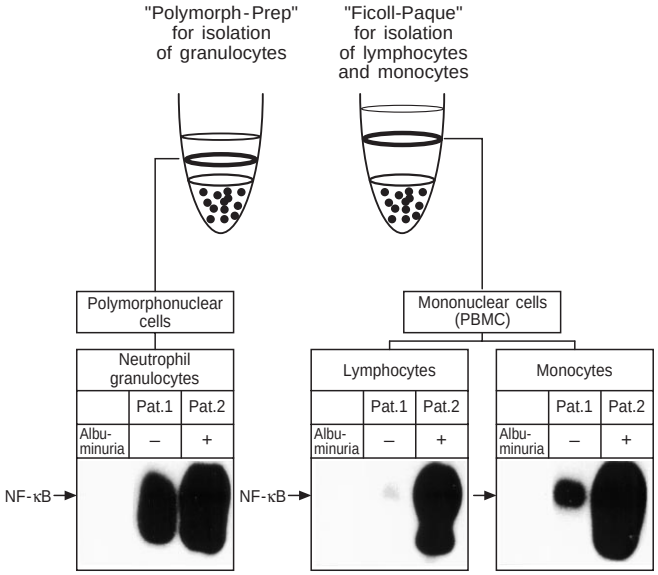
Isolation of PBMC by Ficoll Paque gradient resulted in enrichment of mononuclear cells with less

Table 3. Correlation of PBMC NF-κB binding activity with other variables predicting diabetes mellitus

	R-square	*p*-value
Age	R = 0.0267	0.397
Duration of disease	R = 0.0015	0.8343
HbA_{1c}	R = 0.0002	0.9493
BMI (kg/m^2)	R = 0.0419	0.2694
Cholesterol (mg/dl)	R = 0.0246	0.4164
Systolic blood pressure	R = 0.0200	0.4302
Diastolic blood pressure	R = 0.0282	0.3840

than 3% contamination of neutrophil granulocytes [29, 35, 36]. Cell typing with CD-14, CD-19 and CD-3 antibodies showed that the enriched PBMC fractions contained approximately equimolar amounts of monocytes, B-lymphocytes and T-lymphocytes in all samples tested (Fig. 4). A moderate but statistically not significant switch towards B-lymphocytes was observed in some PBMC fractions of patients with albuminuria (Fig. 4, $p = 0.241$). To define which cell type contributed to the albuminuria dependent NF-κB activation, PBMC fractions were separated into lymphocytes and monocytes [29, 37]. Both fractions were shown to have a strong induction of NF-κB binding activity in PBMC of patients with albuminuria (Fig. 4). Isolated granulocytes showed prominent constitutive NF-κB-binding activity (Fig. 4) which was slightly more pronounced in patients with albuminuria (Fig. 4). Since granulocytes contributed less than 3% to all cells in the PBMC preparation, this weak induction did probably not contribute greatly to the inducible NF-κB signal observed in lymphocytes and monocytes.

To exclude that the NF-κB binding activity determined in EMSA of ex vivo isolated PBMC was caused by cellular activation during the isolation procedure, blood from 14 patients was drawn into syringes containing 3.8% sodium citrate (9:1; vol/vol) supplemented with 2 mmol/l thioctic acid (α-lipoic acid), which previously has been shown to inhibit NF-κB activation in vitro and in vivo [16, 29, 30, 31]. After venipuncture blood smears were prepared, fixed immediately and stained with an antibody reacting exclusively with activated NF-κBp65 [29]. Positivity for activated NF-κBp65 was identified by a dark brown staining within the cells [29, 34]; accordingly only cells with NF-κBp65 staining within the cell were counted as positive [29, 34]. PBMC showing strong activation of NF-κB are marked with arrows. In patients without renal complications mononuclear activation of NF-κBp65 was less as reflected by an average of 36% positively stained cells (Fig. 5a left, Fig. 5b). In contrast, almost all PBMC of patients with albuminuria showed strong reactivity with antibodies for activated NF-κBp65 and an average of 64% PBMC stained positive (Fig. 5a right, Fig. 5b). The difference in staining was statistically significant



Typing of cells present in PBMC-pellets enriched by "Ficoll-Paque"

	Without renal complications (pat.1)	With albuminuria (pat.2)
T-lymphocytes	37%	32%
B-lymphocytes	26%	34%
Monocytes	37%	34%

Fig. 4. Lymphocytes and monocytes contribute to increased NF-κB activation in ex vivo isolated PBMC of patients with albuminuria: granulocytes and mononuclear cells (PBMC) were isolated from two representative patients matched for HbA_{1c} without renal complications and with albuminuria. PBMC were further fractionated by adherence to plasma-coated tissue culture dishes to separate lymphocytes and monocytes. Nuclear extracts were prepared and assayed in EMSA to determine NF-κB binding activity in each of the cell fractions. Radioactive labelled oligonucleotides, spanning the consensus NF-κB-recognition motif, were incubated with equal amounts of nuclear extracts and complexes were separated onto nondenaturing 5% PAA gels. The position of NF-κB is indicated by an arrow. Results of immunhistological typing of cells present in the PBMC pellet using CD-19, CD-3 and CD-14 antibodies are shown below

($p < 0.003$) and confirmed the difference for NF-κB binding activity observed in EMSA.

The antioxidant thioctic acid (α-lipoic acid) has been shown to reduce hyperglycaemia dependent NF-κB binding activity in ex vivo isolated PBMC [29]. To determine whether thioctic acid can also reduce albuminuria mediated NF-κB activation, nine patients with diabetic nephropathy selected at random were treated orally with 600 mg thioctic acid per day for 3 days. Before and after intervention blood samples were collected, PBMC were isolated and NF-κB binding activity was determined as above (Table 2). In parallel, enaldehydes (MDA + 4-HNE) which are said to be plasmatic markers for oxidative stress [44], were measured in plasma samples from the same donor [29] before and after thioctic acid

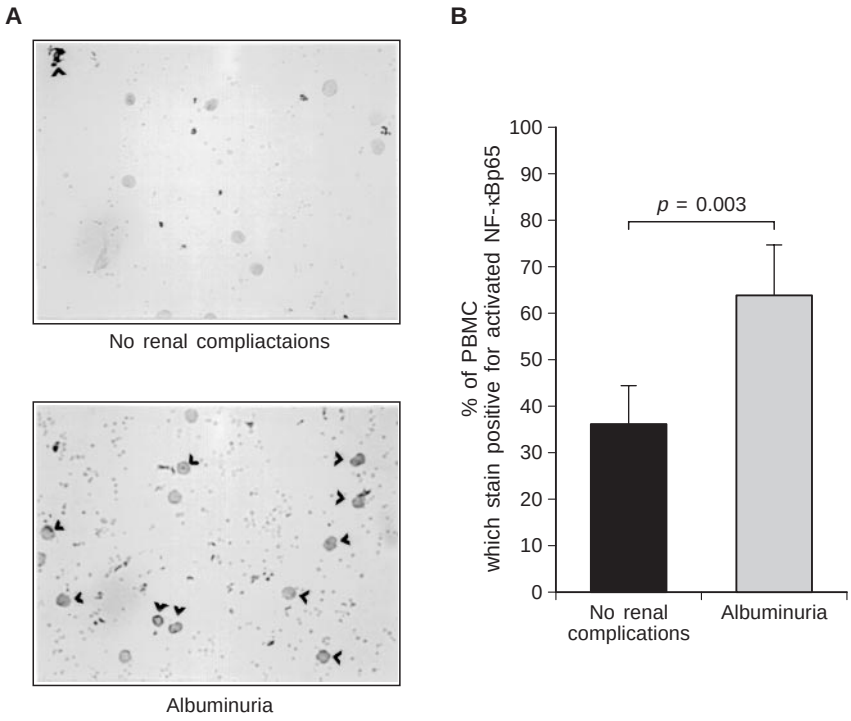


Fig. 5 A, B. Activation of NF-κBp65 is increased in PBMC of patients with albuminuria: **A** Immunhistochemistry for active NF-κBp65 in PBMC of two representative patients without renal complications (top) and with albuminuria (bottom): PBMC and granulocytes fractions were collected (see Materials and methods), combined and seeded onto coverslips, before immunohistochemistry was done using a monoclonal antibody that exclusively reacts with activated NF-κBp65. Positivity for NF-κBp65 is indicated by dark brown color; positive PBMC are indicated by arrows. **B** Compared with patients without renal complications, staining for active NF-κBp65 was significantly increased in PBMC of patients with albuminuria ($p = 0.003$)

supplementation (Table 2). Treatment of 3 days with thioctic acid decreased PBMC derived NF-κB binding activity in all samples tested (Fig. 6 a). The average NF-κB binding activity before thioctic acid supplementation equalled 16 rNF-κBp65 equivalents and was reduced to 9 rNF-κBp65 equivalents after thioctic acid therapy ($p = 0.031$, Fig. 6 b, Table 2). Before thioctic acid treatment the slope indicating the rise in NF-κB binding activity with increasing albuminuria was steeper (Fig. 6 c) than after thioctic acid supplementation (Fig. 6 c). Pearson's correlation for NF-κB binding activity and albuminuria was stronger before (Pearson's coefficient $r = 0.365$, $p = 0.001$) than after (Pearson's coefficient $r = 0.194$, $p = 0.0005$) supplementation with thioctic acid and the difference in NF-κB binding activity in both groups was statistically significant ($p = 0.031$). The thioctic acid mediated decrease in PBMC NF-κB binding activity was paralleled by a 48 % reduction in plasma e-naldehydes (Fig. 6 d), thereby indicating that albuminuria associated NF-κB activation was, at least in part, dependent on oxidative stress.

Plasma thrombomodulin has been described as marker of endothelial activation and endothelial damage which correlates with diabetic microvascular disease [34, 45] and albuminuria [46, 47]. Raised plasma thrombomodulin concentrations have been reported in patients with increased oxidant injury, tubular damage and proteinuria [48]. Accordingly, thrombomodulin plasma concentrations correlated positively with albuminuria (Pearson's coefficient $r = 0.341$, data not shown) and NF-κB binding activity determined in PBMC of 20 of our study subjects (Pearson's coefficient $r = 0.337$; Fig. 7).

Discussion

The pathogenesis of cellular, structural and functional abnormalities in diabetic nephropathy is a multifactorial process associated with persistent hyperglycaemia [49], AGE-formation and deposition [50, 51], hyperlipidaemia [52] and hyperhomocysteinaemia [47]. There is growing evidence, however, that oxidative stress might represent a major common basis for the pathogenesis of renal complications [6–9] by possibly disrupting cellular functions by activation of the transcription factor NF-κB and subsequent coordinated expression of gene products as leucocyte adhesion molecules, cytokines, renal eNOS, MCP-1, endothelin-1 and tissue factor [12, 14–20], all of which are said to contribute to late diabetic complications. Up to date, however, a direct proof of oxidative stress

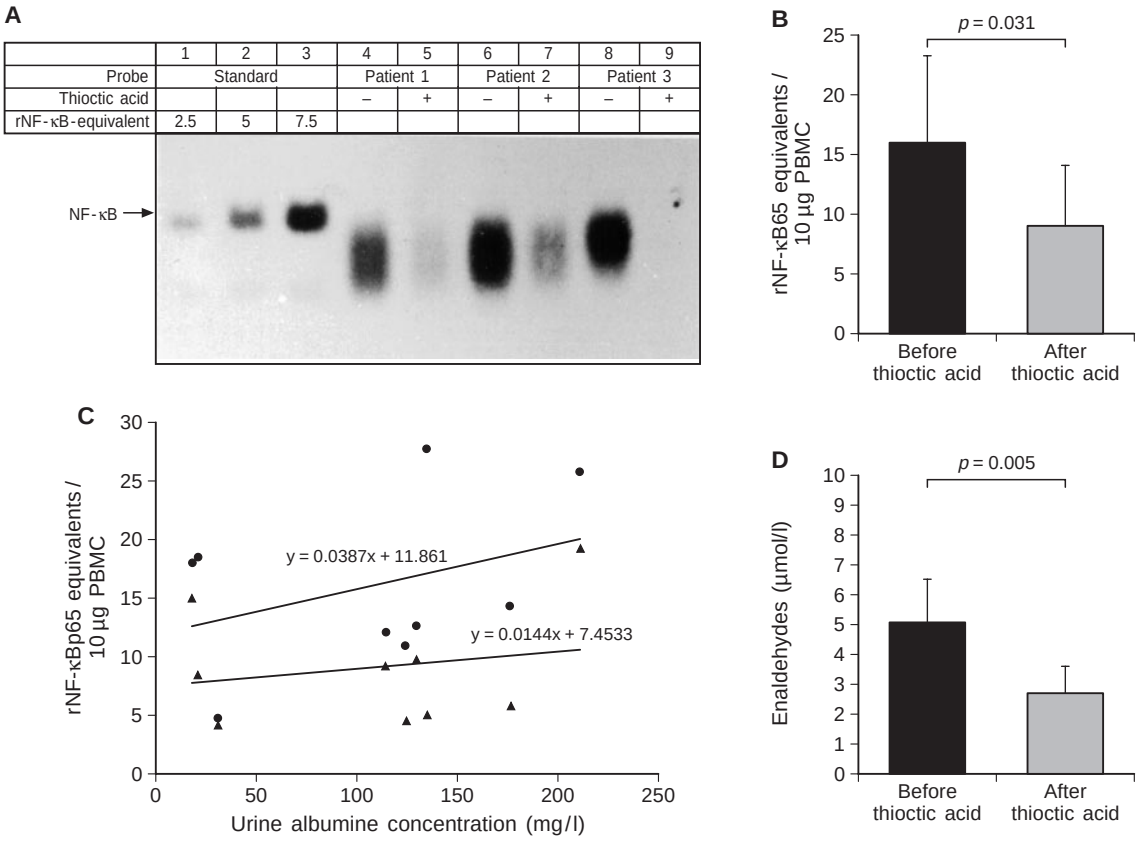


Fig. 6 A–D. NF-κB binding activity in PBMC of patients with albuminuria decreases after treatment with thioctic acid: nine patients selected randomly and matched for HbA_{1c} (Table 2) with diabetic nephropathy received 600 mg of the antioxidant thioctic acid per day orally for 3 days. NF-κB binding activity of ex vivo isolated PBMC (**A–C**) and plasmatic markers of oxidative stress (**D**) were determined in all patients before and after thioctic acid supplementation. **A** NF-κB activation in ex vivo isolated PBMC of three representative patients before and after thioctic acid supplementation. **B** NF-κB binding activity determined for all patients in EMSA was expressed as recombinant NF-κBp65(rNF-κBp65)-equivalent by using the internal standard curve for recombinant NF-κBp65. The average NF-κB binding activity before thioctic acid supplementation equalled 16 rNF-κBp65 equivalents and was reduced to 9 rNF-κBp65 equivalents after thioctic acid supplementation. **C** Correlation of NF-κB binding activity with urine albumine concentrations before (●, $r = 0.365$, $p = 0.001$) and after (▲, $r = 0.194$, $p = 0.0005$) thioctic acid supplementation ($p = 0.031$). **D** Plasmatic markers for oxidative stress (MDA and 4-HNE) were determined in plasma samples of all patients before and after thioctic acid supplementation and are expressed as enaldehyde concentration (µmol/l)

dependent NF-κB activation in patients with diabetic nephropathy is not known.

To define the extent of NF-κB activation in patients with diabetic nephropathy, we used an EMSA based semiquantitative detection system [29] which allowed the determination of NF-κB binding activity in ex vivo isolated PBMC, which – in contrast to kidney biopsies – could be easily obtained during the routine examination of diabetic patients. Compared with patients matched for HbA_{1c} without renal complications, patients with diabetic nephropathy showed a significant increase in NF-κB activation (Fig. 1–3, 6). No correlation of NF-κB was observed with other variables predicting diabetes mellitus such as age, duration of disease, BMI, total cholesterol or blood pressure (Table 3); however, the patient group in this study is too small to carry out a careful multivariate analysis and larger studies will be required to define whether additional variables might influence increased NF-κB activation observed in PBMC of patients with albuminuria.

The correlation of NF-κB with albuminuria was not significantly changed when nephropathy was accompanied by other late diabetic complications such as retinopathy or neuropathy. To evaluate the relative contribution of other late diabetic complications, again much larger studies allowing for multivariate analysis are needed.

To exclude that the EMSA based detection of NF-κB signals in ex vivo isolated PBMC was in part derived from cellular activation during the isolation procedure, peripheral blood smears were prepared and fixed immediately after venipuncture and before staining with an antibody exclusively recognizing activated NF-κBp65 [29, 34]. These conditions almost excluded experimental activation of NF-κB and immunoreactivity was supposed to reflect the in vivo ac-

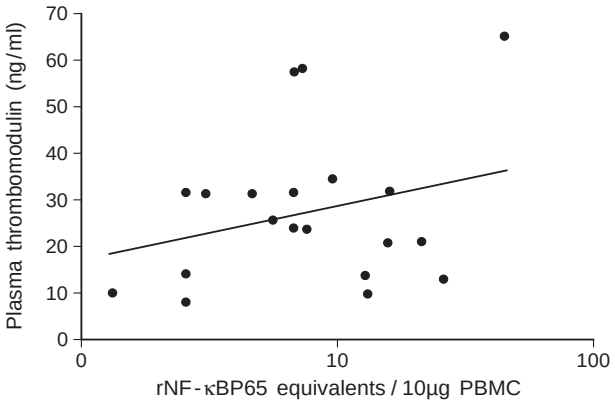


Fig. 7. Plasma thrombomodulin levels correlate NF-κB binding activity: correlation of thrombomodulin plasma concentrations (ng/ml) with NF-κB binding activity (expressed as μg rNF-κBp65 equivalents per 10 μg nuclear extract of PBMC) in 20 patients with diabetes mellitus ($r = 0.337$; $p = 0.05$)

tivation of NF-κB. In patients without renal complications we observed only occasional exactivated NF-κBp65 staining, while mononuclear cells from patients with albuminuria showed increased staining for activated NF-κBp65 (Fig. 5 a,b) and thus validated the results for NF-κB binding activity obtained in EMSA (Fig. 1–3).

Increased NF-κB activation has previously been described in mononuclear cells in the arteriosclerotic plaque [34] and thus, might in part increase the risk for coronary heart disease and arteriosclerosis observed in patients with diabetic nephropathy [49]. In addition, the correlation of thrombomodulin plasma concentrations with albuminuria and NF-κB binding activity (Fig. 7) indicates a parallel activation or damage of endothelial cells. To date it is not known whether NF-κB controlled genes in monocytes and macrophages participate in mediating endothelial cell dysfunction. An oxidative stress dependent general NF-κB activation that affects both mononuclear and endothelial cells might promote further arteriosclerosis since NF-κB controlled endothelial genes include adhesion molecules [12] and the procoagulant tissue factor [25]. Increased plasma concentrations of vascular cell adhesion molecule-1 (VCAM-1) and tissue factor have been reported in patients with albuminuria and microvascular disease [53–55] and increased expression of monocyte procoagulant activity has been shown in Type I diabetic patients with microalbuminuria [56].

Increased NF-κB-activation in monocytes and macrophages might also promote infiltration of macrophages in glomeruli and thus contribute to lipid-induced nephrotoxicity in diabetic nephropathy [57]. NF-κB activation in tissue macrophages might change their ability to effectively degrade and eliminate AGE-macromolecules [50] and therefore support AGE-deposition in renal tissues. The biological function of increased NF-κB binding activity in PBMC of patients with diabetic nephropathy, however, is still not known and further studies will be required.

Recently macrophages from NF-κBp65 deficient mice were shown to be sensitive to TNFα induced cytotoxicity and apoptosis [58]. Anti-apoptotic effects of NF-κB activation in response to endogenous, exogenous, genotoxic, inflammatory and stress-inducing conditions have also been described in other cell types [59]. Thus, NF-κB activation in PBMC of patients with diabetic nephropathy might represent a mechanism to protect monocytes and macrophages from apoptosis, which in turn causes activation of anti-apoptotic genes and the expression of gene products involved in the pathogenesis of late diabetic complications.

To downregulate the excessive NF-κB activation observed in PBMC of patients with diabetic nephropathy it could be difficult to find substances which do not completely inhibit NF-κB and thereby promote apoptosis. In the treatment of inflammatory diseases, glucocorticoids and salicylates have been proven to achieve partial but not complete inhibition of NF-κB [59]. Thioctic acid is another substance which has effectively reduced but not completely suppressed NF-κB activation in vitro [16, 30]. This study shows that the antioxidant thioctic acid reduces, but not eliminates, NF-κB activation in vivo in PBMC of patients with diabetic nephropathy (Fig. 6). Thus, thioctic acid might be suitable to prevent NF-κB dependent cellular changes towards renal end-stage disease, while still allowing protection from cytotoxicity and apoptosis. Whether treatment with thioctic acid might also be suitable to reduce albuminuria in Type I diabetes patients, is currently under investigation.

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