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Intracellular Glutathione Deficiency is Associated with Enhanced Nuclear Factor-κB Activation in Older Non-insulin Dependent Diabetic Patients

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Diabetes mellitus may be associated with intracellular glutathione (GSH) deficiency. Since *in vivo* studies have shown that plasma intracellular GSH plays a key role in regulating the activation of nuclear factor κB (NF-κB), we have investigated the relationship between intracellular thiols (GSH, homocysteine, cysteine and cysteinyglycine) and NF-κB activity in the peripheral blood mononuclear cells (PBMC) of 63 elderly non-insulin dependent diabetes mellitus (NIDDM) patients (28 microalbuminurics and 35 normoalbuminurics) and 30 healthy age- and sex-matched subjects. In addition, we have measured plasma concentrations of these thiol compounds, serum concentrations of interleukin-6 (IL-6) and vascular cell adhesion molecule-1 (sVCAM-1), that are partly dependent on the NF-κB activation, as well as the serum levels of thiobarbituric acid reacting substances (TBARS), as index of lipid peroxidation. Diabetic patients with microalbuminuria (MAB) and normoalbuminuria had NF-κB activity 2.1- and 1.5-fold greater, respectively, than the control group. As compared to normoalbuminuric patients, patients with MAB had significantly higher levels of glycemia, plasma homocysteine, and serum concentrations of TBARS, IL-6 and sVCAM-1 (in all cases, $p < 0.0l$), and significantly lower GSH content in the PBMC ($p < 0.05$). The intracellular GSH in PBMC correlated with NF-κB activation ($r = -0.82$; $p < 0.0001$), serum TBARS ($r = -0.60$; $p < 0.001$), and with fasting glycemia ($r = -0.56$; $p < 0.001$) in patients with MAB, whereas a weaker association between GSH levels in PBMC and NF-κB activation ($r = -0.504$, $p < 0.00l$) was seen in patients without MAB. These results suggest that the decrease of intracellular GSH content in elderly NIDDM patients with MAB is strongly associated with enhanced NF-κB activation, which

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could contribute to the development of increased glomerular capillary permeability and its rapid progression.

Keywords: Glutathione; Thiol compounds; Lipid peroxides (TBARS); Oxidative stress; Nuclear factor κB; Peripheral blood mononuclear cells; Non-insulin-dependent diabetes mellitus; Aging

INTRODUCTION

Many reactions associated with hyperglycemia may acutely and chronically increase the production of free radicals, resulting in an oxidant/antioxidant imbalance.[1] Glutathione (GSH, reduced) is the major intracellular nonprotein thiol compound and has several important functions. First, it is a cofactor for several enzymes and maintains the protein sulphydryl redox status.[2,3] Second, it allows the detoxification of free radicals and oxygen-reactive species involved in several physiological and pathological conditions such as aging,[3–8] diabetes mellitus[9–12] and atherosclerosis.[13] Finally, intracellular GSH plays an important role for the regulation of apoptosis[14] and for inhibiting the activation of nuclear factor-kappa B (NF-κB) by oxygen-free radicals.[15] Data from others[16] and ourselves[17] have shown higher circulating concentrations of interleukin-6 (IL-6) and several acute-phase proteins in type-2 diabetic patients. An increased oxidative stress related to a poor glycemic control seems to be responsible for the state of systemic low-degree chronic inflammatory response that we have found in elderly diabetic patients.[17] One possible mechanism to explain this finding is the activation by the presence of oxygen-free radicals of the NF-κB, which is a critical transcription factor for directing the synthesis and release by phagocytic cells of many proinflammatory molecules, such as cytokines and endothelian-leukocyte adhesion molecules.[18–19]

Recently, we have shown that healthy elderly subjects present a deficiency of intracellular GSH in the peripheral blood mononuclear cells (PBMC) related to a systemic antioxidant/prooxidant imbalance, as compared with young control subjects.[20] To our knowledge, there is no data on intracellular GSH content in elderly non-insulin dependent diabetes mellitus (NIDDM) patients as yet. Since intracellular oxidative stress generated by acute increases in extracellular glucose concentrations,[21] or the presence of advanced glycation endproducts,[22] or both, have been shown to activate NF-κB in cultured bovine aortic endothelial cells, and that oxidative stress leads to vascular dysfunction,[23] we hypothesise that older patients with NIDDM and renal microangiopathy, reflected by the presence of microalbuminuria (MAB), could have higher systemic oxidative stress and lower intracellular GSH concentrations than elderly diabetic patients without MAB and than healthy older subjects. This increased oxidative stress could induce the NF-κB activation and, in turn, increase the production of cytokines and other proinflammatory molecules, resulting in progressive vascular dysfunction and renal microangioapthy.

Thus, the present study analyses the relationship between intracellular GSH levels and NF-κB activity in the PBMC of elderly NIDDM-patients with and without MAB. Circulating concentrations of IL-6 and soluble vascular cell adhesion molecule-1 (sVCAM-1), that are partly dependent on the NF-κB activation, were also measured. Healthy non-diabetic adults of similar age and sex distribution served as controls.

MATERIALS AND METHODS

Subjects

This cross-sectional study was carried out in 63 older patients with type 2 diabetes (36 females and 27 males) diagnosed according to American

Diabetes Association fasting criteria,[24] with a mean duration of disease 7 ± 4.5 years and a lack of other cardiovascular risk factors, except for mild isolated systolic hypertension in 26 patients. The diabetic subjects were divided into two groups: 28 patients had persistent MAB, defined as albumin excretion rate (AER) of 30–300 mg/24 h in two out of three consecutive 24 h collections, and 35 patients had normoalbuminuria (AER <30 mg/24 h). All diabetic patients were treated with isocaloric diet and glibenclamide (5-15 mg/daily).

Clinical data were obtained from the clinic records and confirmed by a direct, interview, physical examination, ultrasound studies of both carotid and femoral arteries using standardised techniques, and fundoscopic evaluation by an ophthalmologist. The urinary albumin concentration was determined by radioimmunoassay. Estimation of creatinine clearance (C_{cr}) was done according to the formula described by Cockcroft and Gault.[25] All participants met the following exclusion criteria: age under 65 years, smoking, overweight (body mass index, BMI >27 kg/m^2), personal history of previous cerebro- or cardiovascular diseases, concomittant illnesses, respiratory, gastrointestinal or genitourinary tract infections referred to a doctor during the last three months, sitting systolic/diastolic blood pressure greater than 150/85 mm Hg, hyperlipidemia (serum cholesterol (≥5.2 mmol/l serum triglyceride ≥1.65 mmol/l), hepatic failure (serum aspartate aminotransferase and/or alanine aminotransferase ≥100 IU/l, prothrombin time ≤60%, total bilirubin ≥60 mmol/l), renal insufficiency (serum creatinine ≥100 mmol/l), macrovascular disease (as judged by pathological changes in the resting electrocardiogram or atherosclerotic lesions of the neck and limb vessels), and proliferative diabetic retinopathy. Fourteen microalbuminuric (50%) and twelve (34%) normoalbuminuric patients had also been treated for isolated systolic hypertension with diuretics at low dose for at least 1 year, but they did not receive any other medication. A third group of 30 healthy subjects (18 females and 12 males) matched for age, gender, and BMI, served as controls. They were apparently healthy as based on their medical history, physical examination and normal values of blood and urine routine laboratory tests. BMI was calculated as weight (kg)/height (m)^2. Informed written consent was obtained from all participants.

Methods

Blood samples were drawn in the morning after an overnight fasting period under standardised conditions. Two blood samples of 5 ml for serum and plasma assays, respectively, were collected and centrifuged without delay at 4°C, and frozen at −70°C until analysis. Serum total cholesterol and triglycerides were measured by standard automatized enzymatic methods (Roche, Switzerland). Fasting serum glucose was measured by the glucose oxidase method on the Beckman glucose analyser and glycosylated haemoglobin-A_{1c} (HbA_{1c}) by a minicolumn chromatography procedure (Bio-Rad, Hercules, CA, USA). Serum levels of lipid peroxides were measured as the reaction products of malondialdehyde with thiobarbituric acid (TBARS) following the method of Griesmaker *et al.* [26] In this reaction, *t*-butyl-4-hydroxyanisole (Sigma, St Louis, MO; USA), in a final concentration of 10 mmol/l, was added to the specimen in order to prevent an artificial autooxidation. Fasting serum vitamin E concentrations were measured by the reverse-phase HPLC method of Lee *et al.*,[27] and results were expressed as vitamin E/total cholesterol molar ratio (mmol/mmol). The specimens for TBARS and vitamin E were stored at −20°C for no longer than 3 weeks before performing assays. Serum levels of IL-6 and the soluble form of the vascular cell adhesion molecule-1 (sVCAM-1) were measured using commercially available enzyme-linked immunoabsorbent assay kits from Medgenix Diagnostics (Fleurus, Belgium) and from R&D Systems (Minneapolis, Minn., USA), respectively. Their

lower detection limits were 0.4 pg/ml and 150 ng/ml, respectively. Inter-assays variabilities were as follows: total cholesterol and triglycerides 2.4%; glucose 1.9%; HbA_{1c} 5.1%; free vitamin E 8.3%; TBARS 6.3%; IL-6 less than 4%, and sVCAM-1 5%.

In addition, peripheral blood mononuclear cells (PBMC) were isolated from freshly drawn heparinized whole blood (10 ml) loaded and centrifugated on Lymphoprep (Nycomed Pharma AS, Oslo, Norway) following the manufacturer's instructions. Cells were counted in Neubauer chambers, being in all experiments the cell viability higher than 95%. The time elapsing between collection of blood sample and aislolation of cells never exceeded 1 h.

HPLC measurement of total intracellular and plasma homocysteine, cysteine, cysteinglycine and glutathione was determined following the method of tributylphosphine/ammonium7-fluorobenzo-2-oxa-1,3-diazide-4-sulphonate (TBP/SBD-F) according to Poele-Pothoff *et al.*[28] For reduction of plasma thiols, 100 µl of plasma and 12.5 µl of internal standard (cysteamine 125 µM) were mixed with 10 µl of 10% TBP in methyl formamide and incubated for 30 min at 4°C. The solution was deproteinized with 150 µl of 10% trichloroacetic acid containing 1 mmol/Na_4EDTA under vortexing, followed by centrifugation for 4 min at 8000*g*. For derivatization, 50 µl of the clear supernatant was mixed with 15 µl of sodium hydroxide 1.55 mol/l, 125 µl borate buffer 0.125 mol/l pH 9.5 containing 4 mmol/l EDTA, and 50 µl SBD-F 1 mg/ml dissolved in borate buffer and incubated for 45 min at 60°C. Twenty µl of the sample was injected. The SBD-F derivatives were eluted isocratically by 0.1 M KH_2PO_4/ 4% acetronitrile (pH 2.1) from a Resolve C18 column (Waters, Milford. MA, USA; 150 × 3.9 mm I.D.), with a flow rate of 1.0 ml/min using a 2690 Alliance chromatography system with automatic injector. A Water 474 scanning fluorescence detector was used for detection of thiol compounds. The fluorescence intensities were measured with excitation at 375 nm and emission at 500 nm. All compounds were quantified with a Waters Millennium chromatography manager.

Nuclear Protein Extraction and Electrophoretic Mobility Shift Assay (EMSA)

Nuclear protein extracts were individually obtained from PBMC of the 63 diabetic patients and 24 out of the 30 healthy control subjects by the method of Montaner *et al.*[29] with our own modifications.[30] Briefly, cells were lysed in cold buffer A (20 mM HEPES [pH 8],1.25% Nonidet P-40, 10 mm KC1, 0.15 mM EGTA, 0.15 mM EDTA, 1 mM dithiothreitol [DTT], 0.2 mM phenylmethylsulfonyl fluoride [PMSF]). Nuclei were pelled by centrifugation at 400*g* and 4°C for 5 min and washed in cold buffer B (20 mM HEPES [pH 8], 50 mM NaCl, 25% glycerol, 0.15 mM EGTA, 0.25 mM EDTA, 1.5 mM $MgC1_2$, 1 mM DTT, 0.2 mM PMSF). After centrifugation, nuclear proteins were extracted by incubation for 30 min in cold buffer C (buffer B but with 400 mM NaCl). Then, nuclear extracts separately obtained from each healthy subject were pooled and used subsequently as single control sample in all assays. This procedure allows us to obtain a reference normal value of NF-κB activity to compare with each of the individual patient samples. Double-stranded oligonucleotide probes of the immunoglobulin gene containing the NF-κB binding site[31] with the following sequences were synthesised:

5′-TCGACGAGCTCGGGACTTTCCGAGC-3′

3′-GCTCGAGCCCTGAAAGGCTCGAGCT-5′

The DNA binding reaction was carried out by incubation of 15 µg of nuclear proteins with ^{32}P-labelled double-stranded oligonucleotides (0.3 ng) in a final volume of 20 µl of reaction mixture (5 mM Hepes [pH 7.8], 50 mM KC1, 0.5 mM DTT, 5 mM $MgC1_2$, 10% glycerol, 0.15 µg of poly [dI-dC]-poly [dI-dC] per µl). The specificity of binding was confirmed by competition with a

400-fold excess of unlabeled κB oligonucleotide in the reaction mixture. After incubation, the samples were loaded onto a 5% polyacrylamide (in 0.5 × Tris-borate-EDTA buffer) gel, which was run at 10 V/cm. The control sample was always loaded onto each gel along with several patient samples. After fixation with 10% acetic acid, the gels were dried and subjected to autoradiography to visualise the NF-κB activity and the specificity of the binding; the radioactivity was quantified using an Instant Imager (Packard). The NF-κB activity value for each patient sample was expressed relative to the NF-κB binding activity obtained in the control sample run in the same gel (taken to be 100%). All samples from each diabetic patient were assayed in two different gels; the final value considered for each sample was the mean of the individual values obtained, which were quite reproducible.

Statistics

Statistical analysis was performed using the SPSS program, version 6.1 for Power Macintosh. Data are expressed as mean ± standard deviation (SD). Analysis of variance (ANOVA) with Scheffe's test was used to calculate differences among the three study groups. Pearson correlation coefficient was used to test the correlation between each two biochemical variables. The Gaussian distribution of the samples was confirmed by the Kolmogorov–Smirnov test. A two-tailed p-value of less than 0.05 was considered significant.

RESULTS

Table I shows the clinical characteristics of the three groups studied. Diabetic patients with MAB had fasting plasma glucose, HbA_{1c} levels, TBARS and plasma total homocysteine concentrations significantly higher than diabetic patients with normoalbuminuria or than aged control subjects. Creatine clearance was similar in aged subjects and normoalbuminuric patients, but significantly lower in diabetics with MAB than in the other two groups. Serum cholesterol and triglycerides (data not shown), serum folic acid, as well as vitamin E/Chol ratio were similar in the three groups and were within normal range. There was a trend, although not statisti-

TABLE I Demographic characteristics and clinical data of elderly non-insulin dependent diabetes mellitus (NIDDM) patients and healthy elderly subjects

	Healthy elderly subject	*Elderly NIDDM patients*	
		With MAB	*Without MAB*
Number of subjects (n)	30	28	35
Gender (Female/Male)	18/12	16/12	20/15
Age (years)	71 ± 5	74 ± 7	70 ± 7
BMI (kg/m^2)	23.7 ± 1.1	24.6 ± 0.9	24.2 ± 0.8
Fasting glucose (mmol/l)	6.0 ± 0.3	8.3 ± 1.1$^{**\#}$	7.1 ± 0.5†
HbA_{1c}(%)	5.3 ± 0.4	6.5 ± 0.9*	5.9 ± 0.9
Serum folate (nmol/1)	10.7 ± 2.0	9.4 ± 1.5	10.2 ± 1.7
Serum creatinine (μmol/1)	88.0 ± 17.6	99.2 ± 17.3*	93.1 ± 18.2
Creatinine clearance (ml/min)	79 ± 6	70.8 ± 5.5*	75.9 ± 4.9
Serum TBARS (μmol MDA/l)	5.3 ± 1.2	8.6 ± 1.2$^{**\#\#}$	6.0 ± 1.2
Vit E/Chol ratio	4.3 ± 0.7	3.8 ± 0.7	4.1 ± 0.8
Plasma Homocysteine (μmol/1)	8.9 ± 2.1	11.8 ± 2.4$^{**\#\#}$	9.5 ± 2.0
Plasma Glutathione (μmol/1)	9.3 ± 3.8	7.4 ± 3.6	7.9 ± 3.4
Plasma Cysteine (μmol/1)	192 ± 34	208 ± 41	201 ± 38

Values are expressed as Means ± SD of the measurements, one-way ANOVA plus Scbeffé test for multiple comparisons; MAB=microalbuminuria; BMI=body mass index; TBARS=thiobarbituric acid reacting substances. $^{*}p \leq 0.05$ and $^{**}p \leq 0.01$ diabetic patients with MAB vs control group; $^{\#}p \leq 0.05$ and $^{\#\#}p \leq 0.01$ diabetic patients with MAB vs patients without MAB. $^{\dagger}p \leq 0.05$ diabetic patients without MAB vs controls.

TABLE II Circulating concentrations of IL-6 and sVCAM-1, NF-κB activity and intracellular content of thiol compounds in the PBMC of elderly NIDDM patients and healthy elderly subjects

	Healthy elderly subjects ($n = 24$)	*Elderly NIDDM patients*	
		With MAB ($n = 28$)	*Without MAB* ($n = 35$)
IL-6 (pg/ml)	2.1 ± 0.9	5.2 ± 1.0$^{**\#\#}$	2.8 ± 1.4
SVCAM-1 (ng/ml)	550 ± 116	726 ± 84$^{*\#\#}$	524 ± 95
NF-κB activity	100	206 ± 24$^{**\#}$	154 ± 21
	Intracellular level of thiol compounds (μmol/mg protein):		
Glutathione	47.3 ± 20.6	27.1 ± 10.6$^{*\#}$	42.6 ± 20.5
Cysteine	6.4 ± 2.0	6.0 ± 1.9	6.3 ± 2.0
Cysteinglycine	0.68 ± 0.53	0.71 ± 0.38	0.68 ± 0.45
Homocysteine	0.07 ± 0.04	0.09 ± 0.05	0.10 ± 0.03

MAB=microalbuminuria; sVCAM-1=vascular cell adhesion molecule-1; NF-κB binding activity in PBMC was expressed as the relative percentage of the control value obtained with nuclear extracts pooled from 24 healthy elderly subjects (100%). $^{*}p \leq 0.05$ and $^{**}p \leq 0.01$ diabetic patients with MAB vs: control group; $^{\#}p \leq 0.05$ and $^{\#\#}p \leq 0.01$ diabetic patients with MAB vs patients without MAB.

cally significant, toward lower plasma GSH concentrations in diabetic patients with MAB.

Table II displays the NF-κB binding activity determined in nuclear protein extracts of PBMC from diabetic patients and control subjects, along with the circulating concentrations of IL 6 and sVCAM-1, two pro-inflammatory molecules that are regulated by NF-κB. Besides, intracellular concentrations of GSH and other related thiol compounds, such as cysteine and the glutathione catabolite cysteinglycine, in the PBMC of the three subject groups, were also shown. Figure 1 shows a representative experiment of EMSA for the NF-κB activity in control subjects and in two diabetic patients, one with microalbuminuria and the other one without it. Elderly diabetic patients with microalbuminuria and normoalbuminuria had NF-κB activity 2.1- and 1.5-fold greater, respectively, than the control group (Table II; Fig. 2 upper panel). Microalbuminuric patients had also significantly higher circulating concentrations of IL-6 and sVCAM-1 than the normoalbuminurics or control subjects. The intensity of NF-κB binding activity tended, although not significantly, to correlate with mean urine albumin concentration ($r = 0.286$; $p = 0.06$). As compared to normoalbuminuric patients and control subjects, patients with microalbuminuria had significantly lower intracellular GSH levels in PBMC (both $p < 0.05$) (Table II; Fig. 2 lower panel); there was a trend toward lower GSH levels in PBMC of the normoalbuminuric patients compared to older controls ($p = 0.089$). Intracellular concentrations of cysteine, cysteinglycine and homocysteine were similar in the diabetic groups and controls.

There was a strong association between NF-κB activation and intracellular GSH levels in the PBMC of elderly NIDDM patients with microalbuminuria ($r = -0.82$; $p < 0.0001$; Fig. 3), and a less remarkable, but yet significant association, between both parameters ($r = -0.504$; $p < 0.001$) in patients without microalbuminuria (Fig. 3). NF-κB activity was not significantly related to fasting glycemia ($r = 0.22$, $p = 0.54$) or glycated haemoglobin ($r = 0.20$, $p = 0.40$) in microalbuminuric patients. The intracellular GSH level in the PBMC correlated with serum TBARS ($r = -0.60$; $p < 0.001$), total plasma homocysteine ($r = -0.49$; $p < 0.0l$) and fasting plasma glucose concentrations ($r = -0.56$; $p < 0.001$) in diabetic patients with microalbuminuria, but it related only to serum TBARS ($r = -0.46$; $p < 0.05$) in patients with normoalbuminuria.

DISCUSSION

In vivo studies have shown that glutathione plays an important role in the detoxification of

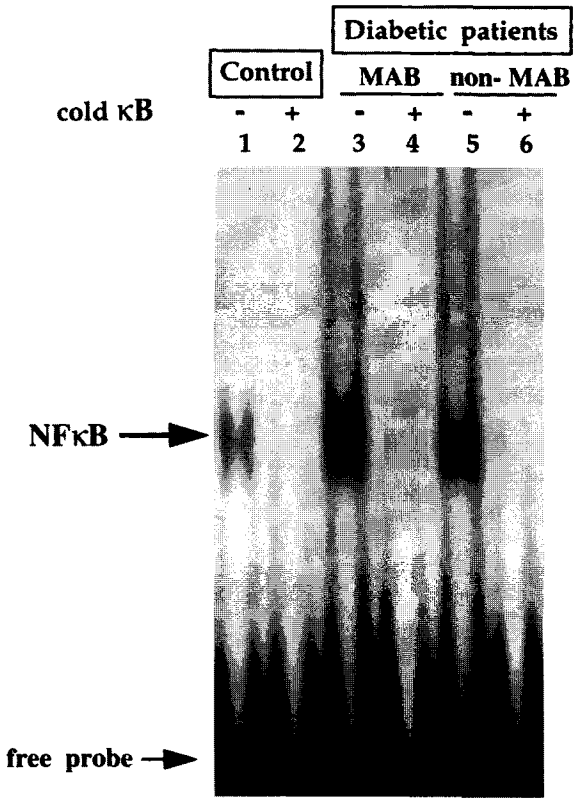

FIGURE 1 Representative electrophoretic mobility shift assay (EMSA) for NF-κB activity in peripheral blood mononuclear cells (PBMC) from controls and diabetic patients. Nuclear protein extracts individually obtained from 24 healthy elderly subjects were pooled and used subsequently as single control sample in all assays. Nuclear protein extract from the control sample (lanes 1 and 2), from a diabetic patient with microalbuminuria [MAB] (lanes 3 and 4), or from a non-microalbuminuric patient [non-MAB] (lanes 5 and 6) were incubated with ^{32}p-labeled κB oligonucleotide, as described in Materials and Methods, and loaded in the same gel. The upper arrow indicates the induced NF-κB band. In lanes 2, 4 and 6, the reaction mixture also contained excess cold unlabeled κB oligonueotide, which completely inhibited binding of the labelled probe. The lower arrow indicates the unbound probe.

reactive oxygen species,[3,4,8] and that intracellular thiols redox status appears to be a critical determinant of NF-κB activation.[15] As compared with young subjects, we have recently reported a deficiency of intracellular GSH in the PBMC of healthy elderly subjects related to a systemic antioxidant/prooxidant imbalance.[20] Since a depletion of cellular GSH, due to an enhanced consumption by oxidative stress, could induce the activation of the NF-κB, in this study we have simultaneously measured the intracellular GSH levels and the NF-κB activity in freshly-isolated PBMC of diabetic patients and healthy older subjects. For the first time, the present study clearly shows that elderly diabetic patients had lower intracellular GSH levels in the PBMC, but significantly higher NF-κB activity, than healthy age- and sex-matched elderly subjects. Moreover, diabetic patients with microalbuminuria had greater deficiency of intracellular GSH and higher NF-κB activity than did patients without microalbuminuria. In addition, NF-κB activity and GSH levels in PBMC were inversely related in the whole group of diabetic patients, and even more markedly in the microalbuminuric patients who also had a worse glycemic control and higher degree of oxidative stress. The absence of any relationship between NF-κB and fasting glycemia or glycated haemoglobin concentrations in diabetic patients with microalbuminuria cannot exclude, indeed, the possibility that long-term elevation in glucose and glycated haemoglobin concentrations may contribute to NF-κB activation, but indicates that intracellular glutathione depletion appears to play a more direct role. In fact, there is increasing evidence that the redox regulation in NF-κB activation may be relevant in the pathogenesis of atherosclerosis,[13] and the aging process.[4,5,8]

Few human studies have demonstrated a link between *in vivo* NF-κB activation and the development of late diabetic complications. In patients with type-1 (insulin-dependent) diabetes, increased oxidative stress generated by poor glycemic control has been shown to induce activation of NF-κB in PBMC.[32] Similarly, increased NF-κB activation has been reported in PBMC from a subset of 21 patients with either type 1 or type 2 diabetes[33] and nephropathy reflected by urinary albumin excretion greater than 100 mg/l. In nine of those patients, a 3-day

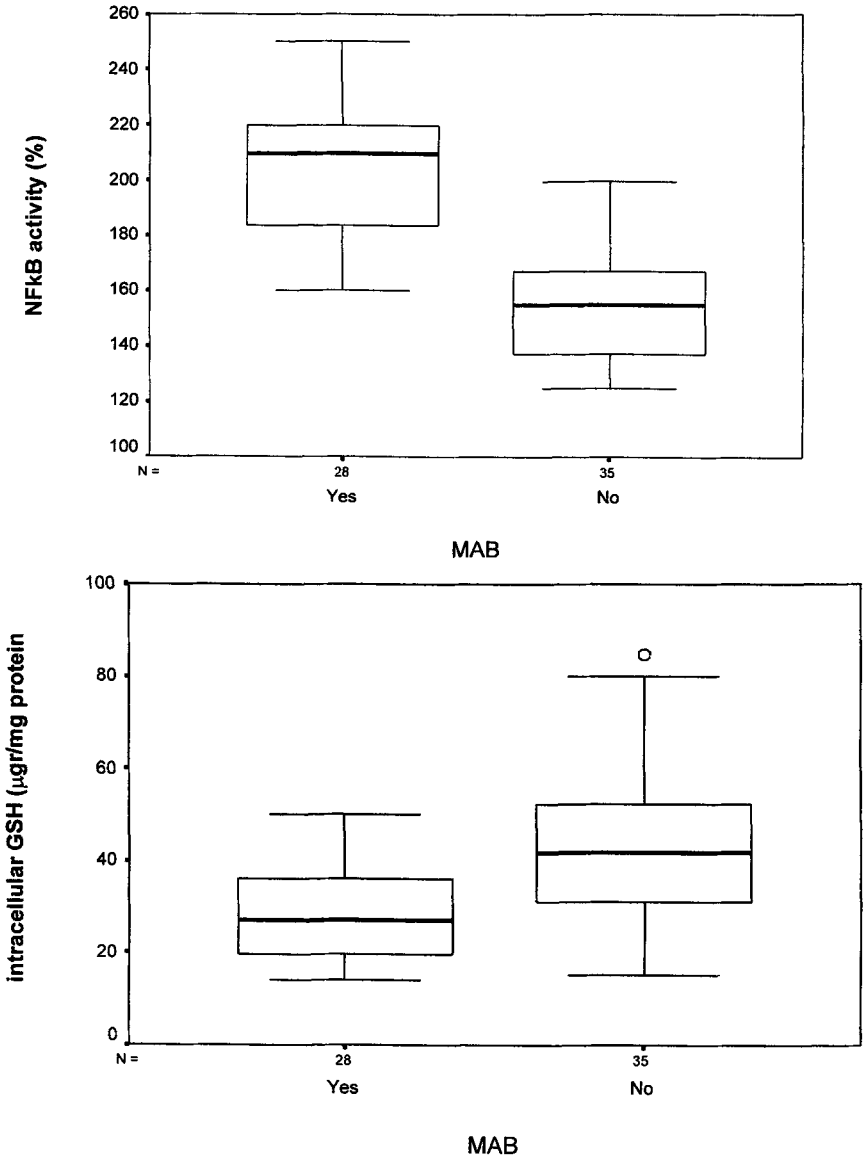

FIGURE 2 Distribution of NF-κB activity (upper panel) and intracellular GSH levels (lower panel) in the PBMC of NIDDM patients according to the presence or absence of microalbuminuria (MAB). Values are represented by Box-whisker plots: horizontal line, median value; box, 25th- and 75th percentiles; whiskers, 5th and 95th percentiles; individual outliers are represented by circles.

treatment with the antioxidant thioctic acid reduced significantly (by 38%) the NF-κB activity, indicating that NF-κB activation might be, in part, dependent on oxidative stress. Our study extends these observations to a larger series of diabetic patients, and reveals that intracellular GSH levels, which actually reflects a dynamic balance between the amount of GSH available and the amount of recently generated oxidant in the cells, significantly influenced the NF-κB activation in the PBMC of type 2 diabetic patients, irrespective of whether or not they had microalbuminuria, although this association was more marked in those patients with microalbu-

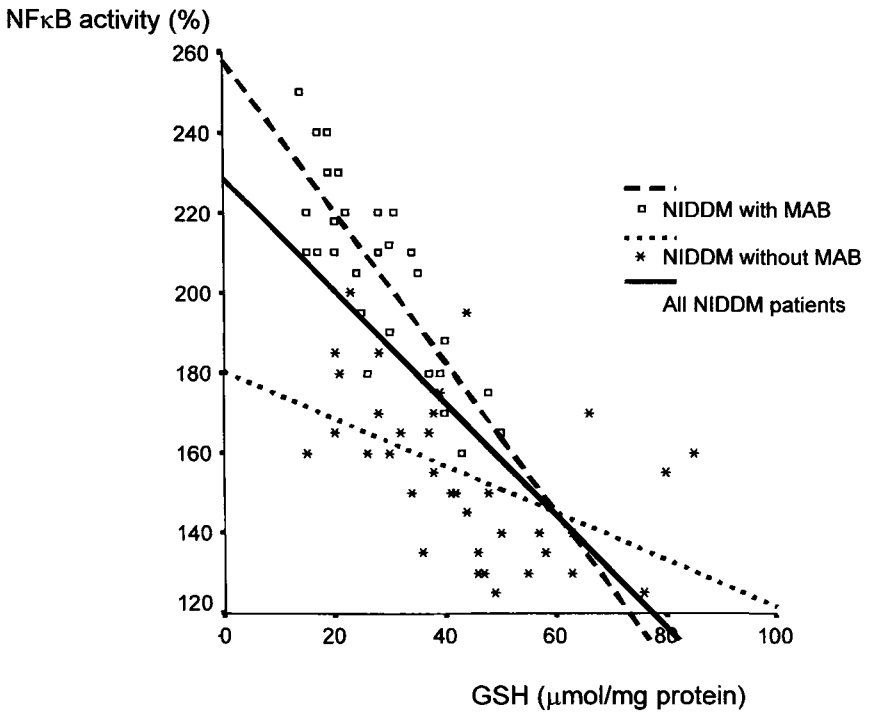

FIGURE 3 Correlation between nuclear factor κB (NF-κB) activity and intracellular glutathione content determined in the PBMC of NIDDM patients with microalbuminuria (dotted line) ($r = -0.819$; $p < 0.0001$), without microalbuminuria (dashed line) ($r = -0.504$; $p < 0.001$), and in the whole group of NIDDM patients (black line) ($r = -0.693$; $p < 0.0001$). The NF-κB activity in patients was expressed as relative percentage of control activity, measured in control subjects (100%).

minuria, most likely because they had the highest oxidant load. Our findings agree with the results from experimental studies showing that intracellular redox reactive substances, such as glutathione, regulate the activation of NF-κB and other nuclear transcription factors simply by scavenging oxidants.[14]

In diabetes, hyperglycemia is accompanied by the formation of advanced glycosylation end products (AGEs) and glycated low-density lipoprotein (LDL) particles that generate oxygen free radicals.[19] Activation of the transcription factor NF-κB by hyperglycemia and AGEs promotes leukocyte adhesion to the endothelium through upregulation of cell surface expression of VCAM-1 and other adhesion molecules.[14,15,34] This is consistent with our findings that both NF-κB activity in PBMC and serum IL-6 and sVCAM-1 concentrations were markedly increased in elderly NIDDM patients with microalbuminuria, compared with normoalbuminuric patients. In addition, these data are in line with previous studies reporting that increased oxidative stress, related to a poor glycemic control, seemed to be responsible for the higher circulating concentrations of IL-6[16,17] and intercellular adhesion molecule-1[35] in NIDDM patients. Thus, NF-κB activation might be an early event in response to increases in glucose, and may contribute to shifting the tone of the arterial wall, leading to increased smooth muscle cell proliferation and vascular complications.[36] The possible role of systemic intracellular GSH depletion and related activation of NF-κB as a mechanism that might worsen the increased glomerular capillary permeability leading to diabetic nephropathy, which is suggested by data in the present study, needs further investigation.

A link between glycemic metabolic control and the content of GSH in erythrocytes[9] and

platelets[12] has been clearly demonstrated in type 1 diabetes mellitus, but the finding of a deficiency of intracellular GSH in the PBMC of older diabetic patients, when compared to healthy elderly subjects, has not been reported as yet. An impairment of glutathione synthesis and thiol transport have been noted in erythrocytes from patients with type 2 diabetes.[10] The observed lack of significant difference in the intracellular PBMC concentrations of cysteine suggests that a decreased availability of this precursor amino acid is not accounted for the GSH deficiency in our diabetic patients compared to aged controls. It appears likely that other causes, such as an increased use of GSH to detoxify peroxides, might contribute to it. In keeping with this view, we have found that the deficiency of intracellular GSH in the PBMC of elderly diabetics was inversely related to the degree of oxidative load expressed by serum levels of TBARS, the by-products of lipid peroxidation. This association could be, at least in part, due to a process of auto-oxidative glycosylation of proteins of cellular membranes that yields oxidising intermediates and resulted in lipid per-oxidation.[23] Besides, the higher concentrations of total plasma homocysteine that we have observed in microalbuminuric patients, which is related to a slight but significant renal function impairment, a finding also noted by others,[37] might further contribute to the production of potent oxygen-reactive species, including the superoxide anion radical and hydroxyl radical, after oxidative metabolism of homocysteine to homocystine and homocysteine thiolactone,[38] which in turn could lead to an oxidation and depletion of the cellular pool of total GSH. Thus, reaction products of hyperglycemia and homocystine might act combined in causing direct endothelial cell cytotoxicity, leading to progressive development of microvascular complications, such as microalbuminuria.

In summary, this study reveals that older NIDDM patients had lower intracellular GSH levels and higher NF-κB activity, determined in freshly isolated PBMC, than healthy age- and sex-matched elderly subjects. Furthermore, diabetic patients with microalbuminuria had much greater deficiency of intracellular GSH and higher NF-κB activity in the PBMC than did patients without microalbuminuria. This deficiency of GSH was related to glycemic levels and the degree of oxidative stress. Thus, our results underscore the need for a better approach to glycemic control in elderly NIDDM patients. The possible role of intracellular GSH depletion and related activation of NF-κB, as a mechanism that might worsen the increased glomerular capillary permeability in NIDDM patients with microalbuminuria, needs further investigation.

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References

[1] Bayness, J.W. and Thorpe, S.R. (1999) "Role of oxidative stress in diabetic complications. A new perspective on an old paradigm", *Diabetes* **48**, 1–9.
[2] Reed, D.J. (1990) "Glutathione: toxicological implications", *Annu. Rev. Pharmacol. Toxicol.* **30**, 603–631.
[3] De la Asunción, J.G., Millán, A., Pla, R., Bruseghini, L., Esteras, A., Pallardo, F.V., Sastre, J. and Viña, J. (1996) "Mitochondrial glutathione oxidation correlates with age-associated oxidative damage to mitochondrial DNA", *FASEB J.* **10**, 333–338.
[4] Sastre, J., Pallardo, F.V., de la Asunción, G.J. and Viña, J. (2000) "Mitochondria, oxidative stress and aging", *Free Radic. Res.* **32**, 189–198.
[5] Cadenas, E. and Davies, K.J. (2000) "Mitochondrial free radical generation, oxidative stress, and aging", *Free Radic. Biol. Med.* **29**, 222–230.
[6] Michelet, F., Gueguen, R., Leroy, P., Wellman, M., Nicolas, A. and Siest, G. (1995) "Blood and plasma glutatwone measured in healthy subjects by HPLC: relation to sex, aging, biological variables, and life habits", *Clin. Chem.* **41**, 1509–1517.
[7] Garcia-Arumí, E., Andreu, A.L., López-Hellin, J. and Schwartz, S. (1998) "Effect of oxidative stress on lymphocytes from elderly subjects", *Clin. Sci.* **94**, 447–452.

[8] Sohal, R.S. and Weindruch, R. (1996) "Oxidative stress, caloric restriction, and ageing", *Science* **219**, 59–63.
[9] Murakami, K., Kondo, T., Ohtsuku, Y., Fujiwara, Y., Shimada, M. and Kawakami, Y. (1989) "Impairment of glutathione metabolism in erythrocytes from patients with diabetes mellitus", *Metabolism* **38**, 753–758.
[10] Yoshida, K., Hiroskawa, J., Tagami, S., Urata, Y. and Kondo, T. (1995) "Weakened cellular scavenging activity against oxidative stress in diabetes mellitus: regulation of glutathione synthesis and eflux", *Diabetologia* **38**, 201–210.
[11] Samiec, P.S., Drews-Botsch, C., Flago, E.W., Kurtz, J.C., Sternberg, P. Jr, Reed, R.L. and Jones, D.P. (1998) "Glutathione in human plasma: decline in association with aging, age-related macular degeneration, and diabetes", *Free Radic. Biol. Med.* **24**, 699–704.
[12] Seghieri, G., Di Simplicio, P., De Giorgio, L.A., Anichini, R., Alberti, L. and Franconi, F. (2000) "Relationship between metabolic glycaemic control and platelet content of glutathione and its related enzymes in insulin-dependent diabetes mellitus", *Clin. Chim. Acta* **299**, 109–117.
[13] Ross, R. (1993) "The pathogenesis of atherosclerosis: a perspective for the 1990s", *Nature* **362**, 801–809.
[14] Dröge, W., Schulze-Osthoff, K., Mihm, S., Galter, D., Schenk, H., Eck, H.-P., Roth, S. and Gmünder, H. (1994) "Functions of glutathione and glutathione disulfide in immunology and immunopathology", *FASEB J.* **8**, 1131–1138.
[15] Staal, F.J.T., Roederer, M., Herzenberg, L.A. and Herzenberg, L.A. (1990) "Intracellular thiols regulate activation of nuclear factor κB and transcription of human immunodeficiency virus", *Proc.Natl Acad. Sci., USA* **87**, 9943–9947.
[16] Pickup, J.C., Mattock, M.B., Chusney, G.D. and Burt, D. (1997) "NIDDM as disease of the innate immune system: association of acute-phase reactants and interleukin-6 with metabolic syndrome X", *Diabetologia* **40**, 1286–1292.
[17] Arnalich, F., Hemanz, A., López-Maderuelo, D., Peña, J.M., Camacho, J., Madero, R., Vázquez, J.J. and Montiel, C. (2000) "Enhanced acute-phase response and oxidative stress in older adults with type II diabetes", *Horm. Metab. Res.* **32**, 407–412.
[18] Barnes, P.J. and Karin, M. (1997) "Nuclear Factor-κB- a pivotal transcription factor in chronic inflammatory diseases", *N. Engl. J. Med.* **336**, 1066–1071.
[19] Collins, T. and Cybulsky, M.I. (2001) "NF-κB: pivotal mediator or innocent bystander in atherogenesis?", *J. Clin. Investig.* **107**, 255–264.
[20] Hernanz, A., Fernández-Vivancos, E., Montiel, C., Vázquez, J.J. and Arnalich, F. (2000) "Changes in the intracellular homocysteine and glutathione content associated with aging", *Life Sci.* **67**, 1317–1324.
[21] Pieper, G.M. and Riaz-ul-Haq (1997) "Activation of Nuclear Factor-(B in cultured endothelial cells by increased glucose concentration: prevention by calphostin C", *J. Cardiovas. Pharmacol.* **30**, 528–532.
[22] Bierhaus, A., Chevton, S., Chevton, M., Hoffmann, M., Quehenberger, P., Illner, T., Luther, T., Berentshtein, E., Tritschler, H., Muller, M., Wahl, P., Ziegler, R. and Nawroth, P.P. (1997) "Advanced glycation end product-induced activation of NF-κB is suppressed by α-lipoic acid in cultured endothelial cells", *Diabetes* **46**, 1481–1490.
[23] Giugliano, D., Ceriello, A. and Paolisso, G. (1996) "Oxidative stress and diabetic vascular complications", *Diabetes Care* **19**, 257–267.
[24] The Expert Committee on the diagnosis and classification of diabetes mellitus(1997) "Report of the Expert Committee on the diagnosis and classification of diabetes mellitus", *Diabetes Care* **20**, 1183–1197.
[25] Cockcroft, D.W. and Gault, M.H. (1976) "Prediction of creatinine clearance from serum creatinine", *Nephron* **16**, 31–41.
[26] Griesmacher, A., Kindhausser, M., Andert, S.E., Schreiner, W., Toma, C., Knoebl, P., Pietschmann, P., Prager, R., Schnack, C., Schernthanert, G. and Mueller, M.M. (1996) "Enhanced serum levels of thiobarbituric- acid-reactive substances in diabetes mellitus", *Am. J. Med.* **98**, 469–475.
[27] Lee, B.L., Chua, H.Y. and Ong, C.N. (1992) "High-performance liquid chromatographic method for routine determination of vitamin A and E, and β-carotene in plasma", *J. Chromatogr.* **581**, 41–47.
[28] te Poele-Pothoff, M.T., van der Berg, M., Franken, D.G., Boers, G.H., Jakobs, C., de Kroon, I.F., Eskes, T.K., Trijbels, J.M. and Blom, H.J. (1995) "Three different methods for the determination of total homocysteine in plasma", *Annal. Clin. Biochem.* **32**, 218–220.
[29] Montaner, S., Ramos, A., Perona, R., Esteve, P., Camero, A. and Lacal, J.C. (1995) "Overexpression of PKC in NIH 3T3 cells does not induce cell transformation nor tumorogenicity and does not, alter NF-κB activity", *Oncogene* **10**, 2213–2220.
[30] Arnalich, F., Garcia-Palomero, E., López, J., Jiménez, M., Madero, R., Renart, J., Vázquez, J.J. and Montiel, C. (2000) "Predictive value of nuclear factor κB activity and plasma cytokine levels in patients with sepsis", *Infec. Immun.* **68**, 1942–1945.
[31] Zabel, U., Schreck, R. and Baeuerle, P.A. (1991) "D.N.A. binding of purified transcription factor NF-κB affinity, specificity, Zn^{2+}dependence, and differential half-site recognition", *J. Biologic. Chem.* **266**, 252–260.
[32] Hoffmann, M.A., Schiekofer, S., Kanitz, M., Klevesath, M.S., Joswig, M., Lee, V., Morcos, M., Trischler, H., Ziegler, R., Wahl, P., Bierhaus, A. and Nawroth, P.P. (1998) "Insufficient glycemic control increases NF-κB binding activity in peripheral blood mononuclear cells isolated from patients with type I diabetes", *Diabetes Care* **21**, 1–7.
[33] Hoffmann, M.A., Schiekofer, S., Isermann, B., Kanitz, M., Henkels, M., Joswig, M., Treusch, A., Morcos, M., Weiss, T., Borcea, V., Abdel Khalek, A.K.M., Amiral, J., Tritschler, H., Ritz, E., Wahl, P., Ziegler, R., Bierhaus, A. and Nawroth, P.P. (1999) "Peripheral blood mononuclear cells isolated from patients with diabetic nephropathy show increased activation of the oxidative-stress sensitive transcription factor NF-κB", *Diabetologia* **42**, 222–232.
[34] Morigi, M., Angioletti, S., Imberti, B., Donadelli, R., Micheletti, G., Figliuzzi, M., Remuzzi, A. and Zoja, C. (1998) "Leukocyte-endothelial interaction is augmented by high glucose concentrations in a NF-κB-dependent fashion", *J. Clin. Investig.* **101**, 1905–1915.
[35] Ceriello, A., Falleti, E., Motz, E., Taboga, C., Tonutti, L., Ezsol, Z., Gonano, F. and Bartoli, E. (1998) "Hyperglycemia-induced circulating ICAM-1 increase in diabetes mellitus: the possible role of oxidative stress", *Horm. Metab. Res.* **30**, 146–149.

[36] Schmidt, A.M. and Stern, D.M. (2000) "Hyperinsulinemia and vascular dysfunction. The role of Nuclear Factor-κB, yet again", *Circ. Res.* **87**, 722–724.
[37] Lanfredini, M., Fiorina, P., Peca, M.G., Veronelli, A., Mello, A., Astorri, E., Dall'Aglio, P. and Craveri, A. (1998) "Fasting and post-methionine load homocyst(e)ine values are correlated with microalbuminuria and could contribute to worsening vascular damage in non-insulin-dependent diabetes mellitus patients", *Metabolism* **47**, 915–927.
[38] Welch, G.N. and Loscalzo, J. (1998) "Homocysteine and atherothrombosis", *N. Engl. J. Med.* **338**, 1042–1050.