Available online at www.sciencedirect.com
ScienceDirect
ELSEVIER
The International Journal of Biochemistry & Cell Biology xxx (2007) xxx–xxx
IJBCB
www.elsevier.com/locate/biocel

Oxidative DNA damage and augmentation of poly(ADP-ribose) polymerase/nuclear factor-kappa B signaling in patients with Type 2 diabetes and microangiopathy

Antonysunil Adaikalakoteswari, Mohan Rema, Viswanathan Mohan, Muthuswamy Balasubramanyam *

Department of Cell and Molecular Biology, Madras Diabetes Research Foundation & Dr.Mohan's Diabetes Specialities Centre, 4 Conran Smith Road, Gopalapuram, Chennai 600086, India

Received 28 January 2007; received in revised form 12 April 2007; accepted 19 April 2007

Abstract

Although oxidative stress and the subsequent DNA damage is one of the obligatory signals for poly(ADP-ribose) polymerase (PARP) activation and nuclear factor-kappa B (NFκB) alterations, these molecular aspects have not been collectively examined in epidemiological and clinical settings. Therefore, this study attempts to assess the oxidative DNA damage and its downstream effector signals in peripheral blood lymphocytes from Type 2 diabetes subjects without and with microangiopathy along with age-matched non-diabetic subjects. The basal DNA damage, lipid peroxidation and protein carbonyl content were significantly ($p < 0.05$) higher in patients with and without microangiopathy compared to control subjects. Formamido Pyrimidine Glycosylase (FPG)-sensitive DNA strand breaks which represents reliable indicator of oxidative DNA damage were also significantly ($p < 0.001$) higher in diabetic patients with (19.41 ± 2.5) and without microangiopathy (16.53 ± 2.0) compared to control subjects (1.38 ± 0.85). Oxidative DNA damage was significantly correlated to poor glycemic control. PARP mRNA expression and PARP activity were significantly ($p < 0.05$) increased in cells from diabetic patients with (0.31 ± 0.03 densitometry units; 0.22 ± 0.02 PARP units/mg protein, respectively) and without (0.35 ± 0.02; 0.42 ± 0.05) microangiopathy compared to control (0.19 ± 0.02; 0.11 ± 0.02) subjects. Diabetic subjects with and without microangiopathy exhibited a significantly ($p < 0.05$) higher (80%) NFκB binding activity compared to control subjects. In diabetic patients, FPG-sensitive DNA strand breaks correlated positively with PARP gene expression, PARP activity and NFκB binding activity. This study provides a comprehensive molecular evidence for increased oxidative stress and genomic instability in Type 2 diabetic subjects even prior to vascular pathology and hence reveals a window of opportunity for early therapeutic intervention.

Keywords: Oxidative DNA damage; Type 2 diabetes; NFκB; PARP; Microangiopathy

1. Introduction

A growing body of evidence suggests that hyperglycemia associated with increased oxidative and nitrosative stress is linked to the pathogenesis of diabetic vascular complications (Evans, Goldfine, Maddux, & Grodsky, 2002). Although reactive oxy-

* Corresponding author. Tel.: +91 44 28359048; fax: +91 44 28350935.
E-mail address: drbalu@mvdsc.org (M. Balasubramanyam).

1357-2725/$ – see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biocel.2007.04.013

gen species (ROS) damage all macromolecules such as lipids, proteins and nucleic acids, DNA is one of the most biologically significant targets of oxidative attack. Increased superoxide generation accompanied by increased nitric oxide generation, favors the formation of strong oxidant peroxynitrate ($OONO^-$), which in turn activates nuclear transcription factor (NFκB) and damages DNA. Excessive DNA damage is an obligatory stimulus for the over-activation of the nuclear enzyme poly(ADP-ribose) polymerase (PARP), which in turn depletes intracellular NAD^+, thus slowing the rate of glycolysis, electron transport, ATP formation and produces ADP ribosylation of many proteins. These processes result in acute endothelial dysfunction in diabetic blood vessels contributing to the development of diabetic complications (Ceriello, 2003).

While there are lots of experimental data to support various oxidative mechanisms, their relevance to clinical diabetes is still largely unexplored. The new concept of oxidative stress, being an important trigger in the onset and progression of diabetes and its complications, is often challenged because intervention studies with classic antioxidants, such as vitamin E, failed to demonstrate any convincing beneficial effects on cardiovascular outcomes. However, these studies point out several flaws in the trials among which much emphasis is on the need for measurement of markers of oxidation to assess the degree of oxidative stress. Among various types of DNA base modifications, 8-hydroxy deoxyguanosine (8-OHdG) has been the most widely studied and is considered as a key biomarker of oxidative DNA damage (Hinokio et al., 1999; Shin et al., 2001). Studies have also shown that activation of PARP and NFκB affects glucose utilization, transcriptional regulation and gene expression via multiple mechanisms, thus indicating PARP/NFκB interface as a novel drug target for therapeutic intervention (Hassa & Hottiger, 2002; Pacher, Obrosova, Mabley, & Szabo, 2005). Since cellular systems under physiological condition are protected by an efficient DNA repairing machinery, we hypothesize that (a) an increase in DNA damage would be demonstrated in subjects with diabetes and its vascular complications and (b) studying downstream effectors of DNA damage (PARP/NFκB) in a clinical setting could provide an additional link between molecular intricacies of oxidative stress and the pathogenesis of diabetes and/or its vascular complications. To test our hypothesis, we assessed the extent of oxidative damage and its relationship to PARP and NFκB modulation in diabetes and microangiopathy along with measurements of glycemic and lipid status in clinically well-characterized groups of subjects.

2. Materials and methods

2.1. Sample selection

Study subjects were recruited from the Chennai Urban Rural Epidemiology Study (CURES), a population-based study in Chennai (formerly Madras) in Southern India. The methodology of CURES has been published elsewhere (Deepa, Pradeepa, & Rema, 2003; Rema, Mohan, Deepa, & Ravikumar, 2004). Details such as age, sex and in diabetic subjects, duration of diabetes and other details of diabetic therapy were recorded and clinical examination was done in all subjects. Subjects undiagnosed for diabetes underwent oral glucose tolerance tests (OGTT) using 75 g of oral glucose load (dissolved in 250 ml of water). Those who were confirmed by OGTT to have 2 h plasma glucose value <7.8 mmol/l (140 mg/dl) were categorized as normal glucose tolerance (NGT) and those with 2 h plasma glucose value >7.8 mmol/l (140 mg/dl) and <11.1 mmol/l (200 mg/dl) were categorized as impaired glucose tolerance (IGT). Diabetics were diagnosed as per the WHO criteria. For the present study we randomly selected (using computer generated random numbers) 40 NGT subjects and 40 diabetic subjects each with and without microangiopathy.

Microangiopathy was diagnosed if nephropathy and/or retinopathy were present. Nephropathy was diagnosed if the patients had either persistent proteinuria (≥500 mg/day) or persistent microalbuminuria (if albuminuria estimated by albumin creatinine ratio (ACR) exceeded 30 μg/mg of creatinine) in the absence of urinary tract infection (Mohan et al., 2000; Varghese, Deepa, Rema, & Mohan, 2001). Retinopathy was assessed using stereoscopic color retinal photography as described earlier (Rema et al., 2004). Briefly, the pupils were dilated using one drop each of phenylepherine 10% and tropicamide 1% into both eyes and the drops were repeated until the best possible mydriasis was obtained. A trained photographer carried out four-field color retinal photography with a Zeiss FF 450 plus camera using 35 mm color transparencies. The photographs were graded against standard photographs of the Early Treatment Diabetic Retinopathy Study (ETDRS) grading system for severity of retinopathy. Hypertension was diagnosed if the subjects had been treated with antihypertensive drugs or had systolic blood pressure (SBP) ≥ 140 mmHg or diastolic blood pressure (DBP) ≥ 90 mmHg. Diabetic subjects without

Please cite this article in press as: Adaikalakoteswari, A., et al., Oxidative DNA damage and augmentation of poly(ADP-ribose) polymerase/nuclear factor-kappa B signaling in patients with Type 2 diabetes and microangiopathy, International Journal of Biochemistry and Cell Biology (2007), doi:10.1016/j.biocel.2007.04.013

microangiopathy were selected on the basis of absence of retinopathy (on retinal photography) or nephropathy (24 h protein excretion <100 mg/day and urinary albumin levels <30 μg/mg creatinine). They also had no history of angina or myocardial infarction and had normal 12 lead resting ECGs and normal peripheral Doppler studies. Informed consent was obtained from all study subjects and the institutional ethics committee approved the study.

2.2. Clinical and biochemical characterization

Physical examination included height, weight, waist and hip measurements using standardized techniques. Blood pressure was recorded in the right arm with a mercury sphygmomanometer (Diamond Deluxe Blood pressure apparatus, Pune, India) while the patients were seated. Two readings were taken 5 min apart and the mean of the two was taken as the blood pressure. A fasting blood sample was taken and serum separated and stored at −70 °C until the assays were performed. Biochemical analyses were done on Hitachi-912 Autoanalyser (Hitachi, Germany) using kits supplied by Roche Diagnostics (Mannheim, Germany). Fasting plasma glucose (GOD–POD method), serum cholesterol (CHOD–PAP method), serum triglycerides (GPO–PAP method) and HDL cholesterol (Direct method–polyethylene glycol-pretreated enzymes) were measured. Low-density lipoprotein (LDL) cholesterol was calculated using the Friedewald formula. Glycated haemoglobin (HbA1C) was estimated by high-pressure liquid chromatography using the Variant machine (Bio-Rad, Hercules, CA, USA). Informed consent was obtained from all study subjects and the study was conducted in compliance with the Helsinki Declaration and approved by the institutional ethics committee of the Madras Diabetes Research Foundation.

2.3. Assessment of intima-medial thickness (IMT)

Intima-media thickness (IMT) of the carotid arteries was determined using a high resolution B mode ultrasonography system (logic 400 GE, Milwaukee, WI, USA) having an electrical linear higher frequency transducer (7.5 MHz) as described previously (Mohan, Ravikumar, ShanthiRani, & Deepa, 1998).

2.4. Isolation of peripheral blood Lymphocytes

Twelve milliliters of fasting peripheral blood was collected from each subject. Two milliliters was used to collect plasma and perform lipid and protein oxidation assays. The rest was compartmentalized for lymphocyte isolation and to perform Comet, PARP activity and NFκB–EMSA (5 ml) and PARP mRNA profiling (5 ml). Lymphocyte isolation was performed as per our previous protocol (Balasubramanyam, Kimura, Aviv, & Gardner, 1993). Briefly, freshly collected peripheral blood was diluted (1:1) with phosphate buffer saline (PBS) and carefully layered on Histopaque-1077 (Sigma–Aldrich, India) gradient and centrifuged at 1600 rpm for 30 min. The buffy-coat interface representing peripheral blood lymphocytes was aspirated, washed three times in PBS (pH 7.4) and used for various studies. FPG-sensitive DNA damage assessment, PARP mRNA expression and activity and NFκB binding assay were determined in a subset of randomly selected subjects ($n = 20$ in each category).

2.5. Comet assay

DNA strand breaks and FPG-sensitive sites were detected in peripheral blood lymphocytes by single cell gel electrophoresis, the comet assay (Singh, McCoy, Tice, & Schneider, 1988). Clear microscope slides were pre-coated with 1% normal melting agarose. For each slide, 100 μl of cell suspension (approximately 10,000 cells) was mixed with 200 μl of 0.5% low melting point agarose, spotted as first layer onto the pre-coated slide and covered with a coverslip. After agarose solidification the coverslip was gently removed; a second layer of 200 μl of normal melting agarose (NMA) was added over the first layer, covered with a coverslip and allowed to solidify. Cover slips were removed and slides were placed in chilled lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris–HCl; pH 10, 1% Triton X-100 and 10% DMSO added just before use) at 4 °C for 1 h. After lysis, the slides were placed on the platform in an electrophoresis tank that contains the pre-chilled (4 °C for at least 1 h) electrophoresis solution (300 mM NaOH, 1 mM EDTA, pH 13). The buffer should just barely cover the slides and was incubated for 30 min at 4 °C before beginning electrophoresis. The electrophoresis was subsequently conducted at 25 V constant voltage and 300 mA for 30 min. Then slides were removed from electrophoresis apparatus and washed with three changes of neutralization buffer in staining jar for 5 min each at 4 °C. Each slide was stained with 75 μl of ethidium bromide (20 μg/ml) and covered with a cover slip. The slides were examined under a fluorescent microscope and analyzed within 3–4 h. Slides were scored using an image analysis system (Comet Imager 1.2.13) attached to a fluorescent microscope (Carl-Zeiss, Germany) equipped with appropriate filter. The microscope was connected to

Please cite this article in press as: Adaikalakoteswari, A., et al., Oxidative DNA damage and augmentation of poly(ADP-ribose) polymerase/nuclear factor-kappa B signaling in patients with Type 2 diabetes and microangiopathy, International Journal of Biochemistry and Cell Biology (2007), doi:10.1016/j.biocel.2007.04.013

a computer through a charge coupled device (CCD) camera to transport images to software for analysis. The final magnification was 400×. Analysis of mean % DNA in the tail, one of the reliable indicators of DNA damage was done using image analysis software. Images from 50 cells (25 from each duplicate slide) were analyzed. To show the reproducibility of our method, we measured DNA damage in peripheral blood lymphocytes from eight subjects on two different occasions. For this, blood samples were taken twice from the same subject on two different occasions; the respective samples were used for the comet assay and checked for correlation ($r = 0.87$) and significance ($p < 0.001$).

2.6. Detection of FPG-sensitive sites (oxidative DNA damage)

Additional DNA breaks formed at sites containing 8-oxo-deoxyguanosine were detected by incubating the DNA with the enzyme Formamido Pyrimidine Glycosylase (FPG) (Collins, Duthie, & Dobson, 1993). After the lysis step in the comet assay, the slides with and without FPG treatment were washed three times (for 5 min each) in enzyme buffer (40 mM Hepes, 100 mM KCl, 0.5 mM EDTA, 0.2 mg/ml BSA; pH 8.0), covered with 100 μl of either buffer or FPG, sealed with a coverslip and incubated for 30 min at 37 °C. Slides were then continued with the electrophoresis till staining and scoring. FPG-sensitive sites were calculated by subtracting the percent DNA in tail obtained in the absence of enzyme from the percent DNA in tail in the presence of enzyme.

2.7. RNA extraction

Total RNA from peripheral blood lymphocytes ($\sim 5 \times 10^6$) was extracted using TRI reagent (Sigma–Aldrich, India). To check the integrity of the total RNA, 1 μg was fractionated on a 1% denaturing agarose gel. RNA concentration was quantified spectrophotometrically and had a 280/260 OD ratio between 1.8 and 2.0.

2.8. RT-PCR

Total RNA (1 μg) was reverse transcribed in a 25 μl reaction containing 5× reaction buffer, 0.2 μg random hexamer primers (Qiagen Inc., USA), 200 units murine leukemia virus reverse transcriptase (GE Healthcare, USA), 2.5 mm dNTPs and 50 units Ribonuclease inhibitor in a Thermocycler (Biorad, USA) (55 min at 37 °C, 5 min at 95 °C) (Dincer, Akcay, Alademir, & Ilkova, 2003).

The PARP mRNA expression PCR was probed using specific primers; their sequence is 5′–3′ AAGCCCTAAAGGCTCAGAAC (nucleotide position 168–187) and TTGGGTGTCTGTGTCTTGAC (465–485). The conditions of amplification were: 94 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min for 24 cycles of amplification. The number of cycles was determined to assure that the amplification occurs in the exponential phase. PCR products were separated by 2% agarose gel electrophoresis. The housekeeping GAPDH PCR products obtained by amplifying primers were used as an internal control.

2.9. Evaluation PARP gene product

The PARP gene expression was quantified using BIO-RAD gel documentation and semi-quantitative analysis using its software. The ratio of PARP to GAPDH PCR product, expressed as peak density, was used as an index of PARP gene expression (in densitometric units).

2.10. Preparation of nuclear extract

Nuclear extracts were prepared as described before (Hofmann et al., 1998). Proteinase inhibitors (leupeptin, pepstatin, aprotinin) were included in each solution at the concentrations suggested by the manufacturer. In brief, 5×10^5 cells were lysed in 400 μl ice-cold buffer A (10 mM Hepes (pH 8.0), 10 mM KCl, 100 μM EDTA, 100 μM EGTA, 500 μM DTT, PI) and incubated on ice for 30 min. After incubation, 3%NP-40 is added and centrifuged for 5 min at 13,000 rpm. The supernatant was discarded and the nuclear pellet was resuspended in 100 μl ice-cold buffer B (20 mM Hepes (pH 8.0), 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 500 μM DTT, PI), incubated on ice for 2 h, vortexing every 10–15 min and centrifuged for 5 min at 13,000 rpm. The supernatant containing nuclear proteins was immediately quick-frozen at −80 °C. Protein concentration was determined using a standard curve with bovine serum albumin (BSA: 2–10 μg/ml) according to the Bradford method.

2.11. Assay of PARP activity

For the assay, 100 μg of nuclear protein was used. PARP activity was measured by incorporation of biotinylated poly(ADP-ribose) onto histone-coated proteins in a 96-well plate using PARP universal colorimetric assay kit (Biotech-India, India), and expressed as units of PARP/mg protein.

Please cite this article in press as: Adaikalakoteswari, A., et al., Oxidative DNA damage and augmentation of poly(ADP-ribose) polymerase/nuclear factor-kappa B signaling in patients with Type 2 diabetes and microangiopathy, International Journal of Biochemistry and Cell Biology (2007), doi:10.1016/j.biocel.2007.04.013

2.12. Electrophoretic mobility shift assay (EMSA)

EMSA was performed as described previously (Hofmann et al., 1998). Binding of NFκB to 5′-radiolabelled NFκB consensus oligonucleotide (5′-AGT TGA GGG GAC TTT CCC AGG C-3′; approximately 50,000 cpm) was performed using 10 μg of nuclear extract in 20 μl of 5× binding buffer (50 mM Tris, pH 7.5, 250 mM NaCl, 5 mM DTT, 5 mM EDTA, 25% glycerol) containing 1 μg of poly(dI–dC) and 200 nCi of ^{32}P-labelled probe incubated for 20 min at room temperature. The binding complex was separated on a 5% non-denaturing polyacrylamide gel and electrophoresed in 1× TBE buffer (1 M Tris, 0.9 M boric acid, 0.01 M EDTA, pH 8.0) for 3 h at 150 V. The specificity of the complex was checked using 10 ng of anti-p65 and anti-p50 antibodies (Santa Cruz Biotechnology Inc., CA, USA), forming a new larger DNA–protein–antibody complex. The antibody was incubated with the nuclear protein 30 min before the addition of the probe. For competition studies, a cold competitor was added at 50–100-fold molar excess to the binding mixture, 10 min before the addition of the ^{32}P-labelled probe. Following electrophoresis, the gel was dried under vacuum (at 70 °C for 50 min) and exposed to an X-ray film. The complexes were quantified using BIORAD gel documentation and semi-quantitative analysis was done using its software. The extent of NFκB binding to the consensus NFκB probe was set at 100% for the control group.

2.13. Lipid peroxidation

Plasma level of malonodialdehyde (MDA), a marker of lipid peroxidation was measured by thiobarbituric acid reactive substances (TBARS) by fluorescence methodology (Adaikalakoteswari, Balasubramanyam, & Mohan, 2005; Yagi, 1976).

2.14. Protein carbonyls

Carbonyl content was evaluated by the 2,4-dinitrophenylhydrazine (DNPH) assay (Adaikalakoteswari, Balasubramanyam, Rema, & Mohan, 2006; Levine et al., 1990).

2.15. Statistical analysis

Comparisons between groups were performed using unpaired Student's *t*-test. The criterion for significance is $p < 0.05$. Pearson correlation was used to compare the correlation between DNA damage estimate and other variables. All the analyses were age adjusted. Risk variables that had significant association with oxidative DNA damage (FPG-sensitive sites) on univariate regression, were included as independent variables in multiple linear regression analysis. Care was taken to avoid inter-correlated variables in the regression equation. All analysis was done using Windows-based SPSS statistical package (Version 10.0, Chicago, IL).

3. Results

Table 1 shows the clinical characteristics of study subjects. None of the diabetic patients had ketonuria or any history of diabetic ketosis. Of the 80 Type 2 diabetic subjects, 65% were on oral hypoglycemic agents (OHA) and others (35%) were on OHA plus insulin. Diabetic patients with and without microangiopathy had significantly higher fasting plasma glucose, HbA1c, systolic and diastolic blood pressure, serum cholesterol, triglycerides and carotid IMT compared to control subjects.

The mean (±S.E.) levels of thiobarbituric acid reactive substances (TBARS) and protein carbonyls (PCO), respectively were significantly ($p < 0.05$) higher in diabetic patients with (19.24 ± 1.6 nM/ml; 0.87 ± 0.04 nm/mg protein) and without microangiopathy (17.84 ± 1.3; 0.81 ± 0.06) compared to control subjects (10.04 ± 0.8; 0.6 ± 0.02) (Table 1). The basal DNA damage (percentage of DNA in the tail) (mean ± S.E.) was significantly ($p < 0.05$) higher in diabetic patients with (21.34 ± 1.4) and without (22.36 ± 1.6) microangiopathy compared to control subjects (4.29 ± 1.04) (Fig. 1a). The FPG-sensitive DNA-strand breaks which represent a reliable marker of "oxidative DNA damage" (expressed as percent of DNA in the tail (mean ± S.E.)) were also significantly ($p < 0.001$) higher in diabetic patients with (19.41 ± 2.5) and without microangiopathy (16.53 ± 2.0), when compared to control subjects (1.38 ± 0.8) (Fig. 1b).

Correlation analysis showed that both in control and diabetic subjects, the basal DNA damage were significantly ($p < 0.05$) correlated with HbA1c, cholesterol and triglyceride levels. While the oxidative DNA damage showed no correlation with clinical parameters in control subjects, in diabetic patients they were significantly and positively correlated to duration of diabetes ($r = 0.53$; $p = 0.001$), fasting ($r = 0.44$; $p = 0.005$) and post-prandial ($r = 0.54$; $p = 0.001$) plasma glucose, HbA1c ($r = 0.34$; $p = 0.032$), serum cholesterol ($r = 0.35$; $p = 0.025$), triglycerides ($r = 0.31$; $p = 0.049$), IMT ($r = 0.41$; $p = 0.008$), TBARS ($r = 0.33$; $p = 0.037$) and PCO ($r = 0.37$; $p = 0.019$) levels and negatively correlated to creatinine clearance ($r = -0.47$; $p = 0.007$).

Please cite this article in press as: Adaikalakoteswari, A., et al., Oxidative DNA damage and augmentation of poly(ADP-ribose) polymerase/nuclear factor-kappa B signaling in patients with Type 2 diabetes and microangiopathy, International Journal of Biochemistry and Cell Biology (2007), doi:10.1016/j.biocel.2007.04.013

Table 1
Clinical characteristics of the study subjects

Parameters	Control ($n=40$)	Type 2 diabetes without microangiopathy ($n=40$)	Type 2 diabetes with microangiopathy ($n=40$)
Age (years)	45 ± 10	52 ± 9[a]	54 ± 9[a]
Sex (male %)	40	52	48
Duration of diabetes (years)	0	6 ± 6	9 ± 6[b]
Fasting plasma glucose (mmol/l)	88 ± 8	176 ± 8[a]	205 ± 66[a]
Glycated hemoglobin (%)	5.7 ± 0.6	8.9 ± 2.5[a]	9.5 ± 2.3[a]
Body mass index (kg/m^2)	23 ± 4	24 ± 5	24 ± 3
Systolic blood pressure (mmHg)	118 ± 19	124 ± 25	134 ± 25[a]
Diastolic blood pressure (mmHg)	72 ± 11	75 ± 13	80 ± 12[a]
Serum cholesterol (mmol/l)	181 ± 32	197 ± 45	222 ± 60[a]
Serum triglycerides (mmol/l)	110 ± 49	183 ± 113	242 ± 208[a]
Serum HDL cholesterol (mmol/l)	43 ± 11	44 ± 10	41 ± 10
Serum LDL cholesterol (mmol/l)	116 ± 27	114 ± 35	121 ± 57
Cholesterol:HDL ratio	4.4 ± 1.0	4.6 ± 1.2	5.3 ± 1.4[a,b]
Intima-media thickness (mm)	0.76 ± 0.2	0.87 ± 0.2[a]	0.93 ± 0.2[a]
Creatinine clearance (ml/(min m^2))	89 ± 18	90 ± 25	83 ± 28
TBARS (nM/ml)	10.0 ± 5.1	17.8 ± 8.5[a]	19.2 ± 9.8[a]
PCO (nM/mg protein)	0.60 ± 0.1	0.81 ± 0.4[a]	0.87 ± 0.3[a]

Values are expressed as mean ± S.D.
[a] $p<0.05$ compared to controls.
[b] $p<0.05$ compared to Type 2 diabetes without microangiopathy.

Fig. 2a is an illustration of mRNA gene expression patterns of PARP and GAPDH in the study subjects. PARP mRNA expression was significantly ($p<0.05$) increased in diabetic patients with (0.31 ± 0.03 densitometry units) and without (0.35 ± 0.02) microangiopathy compared to control subjects (0.19 ± 0.02) (Fig. 2b). Concurrent to mRNA findings, PARP activity was also significantly ($p<0.05$) increased in diabetic subjects (0.42 ± 0.05) compared to controls (0.11 ± 0.02) (Fig. 2c). Though PARP activity in diabetes subjects with microangiopathy (0.22 ± 0.02) was lower than patients without microangiopathy, it was significantly ($p<0.05$) higher than the activity levels seen in the control subjects. NFκB binding activity as measured from the EMSA technique is illustrated in Fig. 3a. Diabetic subjects with and without microangiopathy exhibited a significantly ($p<0.05$) higher (80%) NFκB binding activity compared to control subjects (Fig. 3b). In diabetic patients, oxidative DNA damage also correlated positively with PARP gene expression ($r=0.476$; $p=0.002$) (Fig. 4a), PARP activity ($r=0.320$; $p=0.044$) (Fig. 4b) and NFκB binding activity ($r=0.490$; $p=0.001$) (Fig. 4c). When age, sex, smoking, presence of diabetes, HbA1c, cholesterol, triglycerides, creatinine clearance, IMT, TBARS, PCO, NFκB binding activity, PARP gene expression and activity, were included as potential independent variables in the forward regression analysis, presence of diabetes, PARP activity, IMT, and creatinine clearance remained

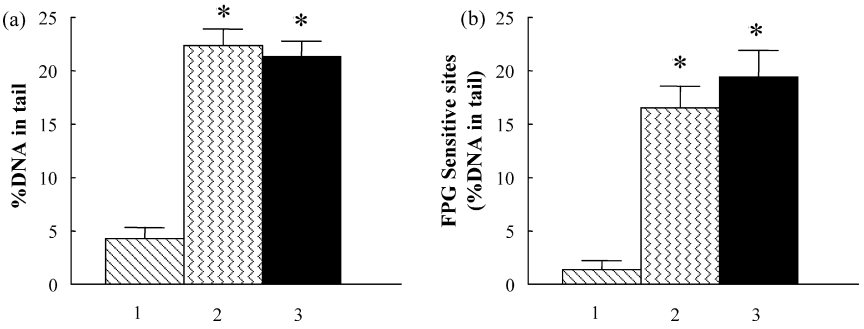

Fig. 1. (a) Basal DNA damage (mean ± S.E.) in (1) control subjects (4.29 ± 1.04), (2) diabetic subjects without (22.36 ± 1.6) and (3) with microangiopathy (21.34 ± 1.5). (b) FPG-sensitive DNA strand breaks (mean ± S.E.) in (1) control subjects (1.38 ± 0.85), (2) diabetic subjects without (16.53 ± 2.0) and (3) with microangiopathy (19.41 ± 2.5). *$p<0.05$ compared to control values.

Please cite this article in press as: Adaikalakoteswari, A., et al., Oxidative DNA damage and augmentation of poly(ADP-ribose) polymerase/nuclear factor-kappa B signaling in patients with Type 2 diabetes and microangiopathy, International Journal of Biochemistry and Cell Biology (2007), doi:10.1016/j.biocel.2007.04.013

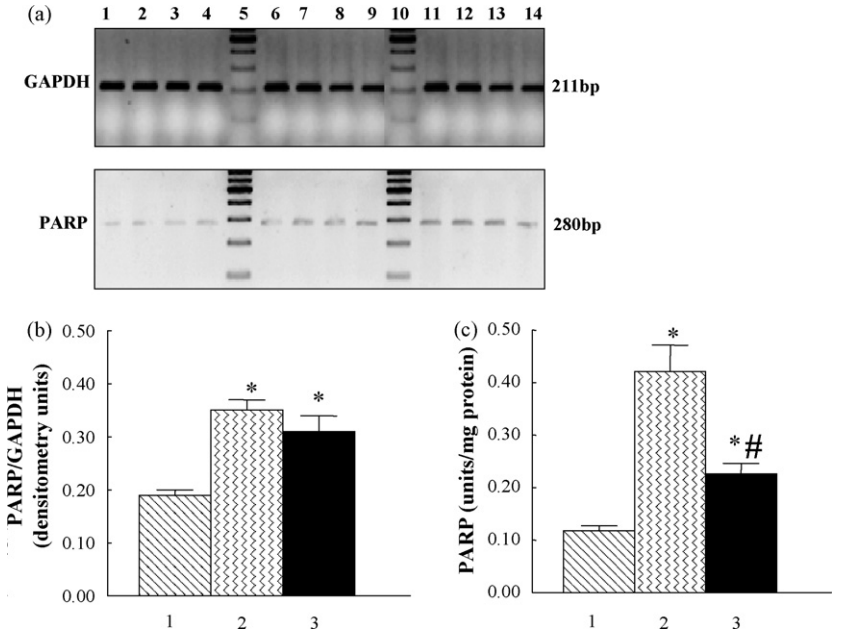

Fig. 2. (a) Representative illustration of gene expression patterns of PARP and GAPDH in control subjects (lanes 1–4), diabetic subjects without microangiopathy (lanes 6–9) and diabetic subjects with microangiopathy (lanes 11–14). Lanes 5 and 10 show DNA ladder (100–700 bp). After densitometric analysis, expression of PARP mRNA was adjusted for the expression of the housekeeping gene GAPDH. (b) Estimated PARP mRNA expression (mean ± S.E.) for (1) control subjects, (2) diabetic subjects without and (3) with microangiopathy were 0.19 ± 0.02, 0.35 ± 0.02, 0.31 ± 0.03, respectively. (c) PARP activity (mean ± S.E., units/mg protein) for (1), (2) and (3) were 0.11 ± 0.02, 0.42 ± 0.05, 0022 ± 0.02, respectively. *$p < 0.05$ compared to control values, # $p < 0.05$ compared to values in diabetic subjects without microangiopathy.

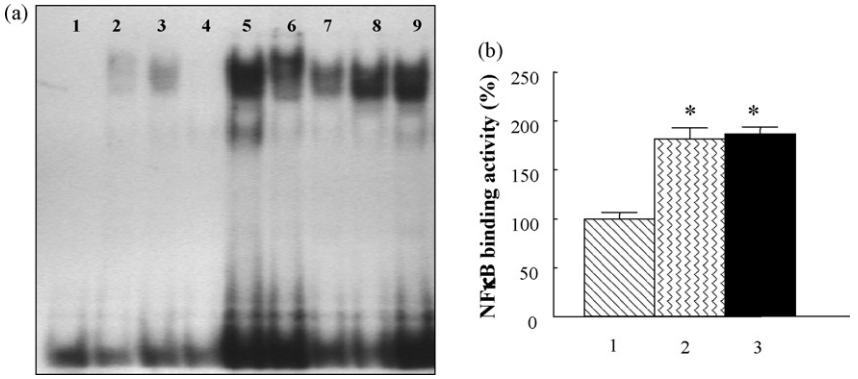

Fig. 3. (a) Representative illustration of NFκB binding activity by EMSA technique. Lanes 1 and 4: free probe; lanes 2 and 3: control subjects; lanes 5–7: Diabetic subjects without microangiopathy; lanes 8 and 9: diabetic subjects with microangiopathy. (b) Cumulative data after densitometric analysis for NFκB binding activity: estimated NFκB binding activity in (1) control subjects (set at 100%), (2) diabetic subjects without (182%) and (3) with microangiopathy (186%).

in the final regression equation as risk variables arising out of oxidative DNA damage.

4. Discussion

Oxidative stress has been implicated in the pathogenesis of various chronic diseases including Type 2 diabetes and its complications. Evidence suggests a link among hyperglycemia, oxidative stress and reduced antioxidant potential, but their relationship in clinical diabetes is unclear. While intervention studies with classic antioxidants such as vitamin E failed to demonstrate convincing beneficial effects (Balasubramanyam, 2006; Ceriello, 2003), they do recommend that assessment of oxidative damage should be done in epidemiological and clinical studies. Despite a few studies, the subject of oxidative stress in diabetes and its complications has not been addressed in an epidemiological setting. The

Please cite this article in press as: Adaikalakoteswari, A., et al., Oxidative DNA damage and augmentation of poly(ADP-ribose) polymerase/nuclear factor-kappa B signaling in patients with Type 2 diabetes and microangiopathy, International Journal of Biochemistry and Cell Biology (2007), doi:10.1016/j.biocel.2007.04.013

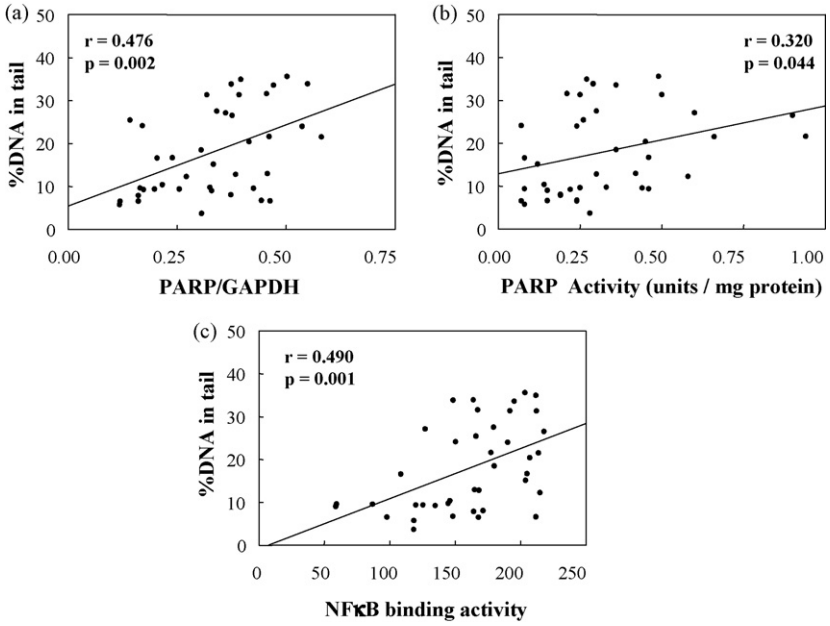

Fig. 4. Correlation of FPG-sensitive DNA strand breaks with (a) PARP mRNA expression ($r = 0.476$; $p = 0.002$), (b) PARP activity ($r = 0.320$; $p = 0.044$) and (c) NFκB binding activity ($r = 0.490$; $p = 0.001$) in diabetic subjects.

absence of epidemiologic data on oxidative damage in normal human populations represents a serious gap in our knowledge about the distribution, correlates, and causative factors of oxidative damage. Therefore, the present study assumes significance for the following reasons. First, oxidative damage to the macromolecules (lipid, protein and DNA) was comprehensively assessed in subjects from the epidemiological programme. Secondly, the study establishes a significant correlation between oxidative DNA damage and a number of clinical phenotypes associated with Type 2 diabetes. Thirdly, molecular evidence for increased oxidative stress in clinical diabetes and microangiopathy was provided through a comprehensive analysis of oxidative DNA damage and augmentation of PARP/NFκB signals.

Research on the cellular effects of hyperglycemia has elucidated several molecular defects among which oxidative damage appears to represent a central abnormality (Brownlee, 2001; Ceriello, 2003). Increased lipid and protein oxidation have been demonstrated in both experimental and clinical diabetes studies, with more recent studies pointing to F2-isoprostane and nitrotyrosine emerging as useful biomarkers of oxidative stress (Morrow, 2005; Pacher et al., 2005). Several studies have also shown increased oxidative DNA damage (Choi et al., 2005; Dincer et al., 2003; Pitozzi, Giovannelli, Bardini, Rotella, & Dolara, 2003; Sampson, Winterbone, Hughes, Dozio, & Hughes, 2006) and accumulation of 8-OHdG (Hinokio et al., 1999; Shin et al., 2001; Speit, Schutz, Bonzheim, Trenz, & Hoffmann, 2004) in patients with diabetes suggesting the involvement of hyperglycemia in oxidative DNA damage. Increased oxidative damage related to mitochondrial DNA (mtDNA) alterations was also reported in Type 2 diabetes (Speit et al., 2004).

In many studies, DNA damage was assessed as DNA strand breaks to look for endogenous oxidative damage to DNA. However there are some limitations to this approach: (a) breaks can arise in a variety of ways unrelated to oxidation and (b) breaks are quite rapidly repaired by cells (Collins, 2004; Lin et al., 2005; Speit et al., 2004). In our study, we have used FPG to recognize altered purines (including 8-oxo-guanine, the principal substrate for FPG in vivo) and thereby estimated the oxidative DNA damage. Oxidative DNA damage (FPG-sensitive DNA strand breaks) in diabetic patients was very well correlated to poor glycemic control (in terms of fasting and post-prandial hyperglycemia and HbA1c) suggesting that glucose toxicity may be the main culprit contributing to enhanced oxidative stress. Since patients with high blood glucose and high lipid levels are at an increased risk for vascular damage, the results also suggest that in the presence of chronic hyperglycemia and hyperlipidemia, any increase in oxidative DNA damage might serve as a reliable indicator of increased predilection to diabetic vascular complications.

The association of FPG-sensitive sites with lipid peroxidation and protein carbonyl levels among the

Please cite this article in press as: Adaikalakoteswari, A., et al., Oxidative DNA damage and augmentation of poly(ADP-ribose) polymerase/nuclear factor-kappa B signaling in patients with Type 2 diabetes and microangiopathy, International Journal of Biochemistry and Cell Biology (2007), doi:10.1016/j.biocel.2007.04.013

diabetic subjects in our study further substantiates the inter-relationship between lipid-, protein- and nucleic acid-damage and induction of the oxidative stress (Choi et al., 2005). Additionally we also observed that both oxidative DNA damage and intimal thickening were greater in diabetics. This is in consistent with the findings in which oxidative DNA damage was associated with human atherosclerotic plaques that promote cell proliferation, hypertrophy, growth arrest and/or apoptosis (Irani, 2000; Kunsch & Medford, 1999). Recently, Demirbag, Yilmaz, & Kocyigit (2005) also reported increased DNA damage with severity of CAD and its risk factors.

In addition to oxidative DNA damage, our study revealed increased PARP mRNA expression and activity and augmented NFκB signaling in patients with Type 2 diabetes and microangiopathy. Evidence implicates that PARP is involved in the onset of pathophysiological diseases at two different levels: (1) PARP acting as a sensor of DNA damage in cells and (2) PARP acting as transcriptional co-activator of NFκB (Hassa & Hottiger, 2002). Recent studies have delineated a role of PARP-1 activation in the pathogenesis of diabetes and diabetic complications including cardiovascular dysfunction (Pacher et al., 2002), nephropathy (Wahlberg, Carlson, Wasserman, & Ljungqvist, 1985), neuropathy (Ilnytska et al., 2006; Li et al., 2004) and retinopathy (Zheng, Szabo, & Kern, 2004).

It is suggested that increased ROS play a critical role in signaling the cellular inflammatory response by activating the redox-sensitive transcription factor, NFκB (Kabe, Ando, Hirao, Yoshida, & Handa, 2005). In our study, NFκB binding activity was significantly increased in cells from patients with diabetes and microangiopathy. More importantly, oxidative DNA damage as represented by FPG-sensitive sites was positively correlated with three different molecular parameters viz., PARP mRNA expression, PARP activity and NFκB binding activity. This emphasizes a tight link between oxidative DNA damage and augmented PPAR/NFκB signaling in patients with diabetes. Increased DNA binding affinity of NFκB in retinal endothelial cells exposed to high glucose was demonstrated to be mediated via PARP activity (Zheng et al., 2004) and binding of p50 subunit to DNA was shown poly(ADP) ribosylation-dependent (Chang & Alvarez-Gonzalez, 2001). In addition, reduced expression of NFκB dependent pro-inflammatory mediators was reported in PARP-1(−/−) mice (Shall & de Murcia, 2000). Although DNA damage is considered as an obligatory signal for PARP activation, it may also occur in the absence of DNA damage through the ERK2 pathway (Cohen-Armon et al., 2007). Alternatively, the pro-inflammatory cytokines often associate with diabetes may also stimulate ERK/NFκB signaling (Larsen et al., 2005). Therefore, it appears that the NFκB/PARP interface might be a potential target to interrupt the vicious cycle of oxidative stress and for the development of new combinations of drugs disrupting specific protein–protein interactions.

While a role for oxidative stress has been demonstrated in the development of microangiopathy in diabetic patients, the oxidative DNA damage seen in diabetic subjects with microangiopathy was not significantly higher compared to that observed in diabetic subjects without microangiopathy. It is unlikely to be explained as a feedback control by DNA repair enzymes because vascular complications were very often related at the cellular level to augmentation of poly(ADP) ribosylation and NFκB events. The plausible explanation for our results in diabetes patients with microangiopathy may be related to drug regimen in these patients in addition to glucose-lowering agents, which could modulate DNA damage and interfere with PARP/NFκB signals (Martin-Ventura et al., 2005; Ohga et al., 2007; Schmeisser et al., 2004; Shin et al., 2005). Alternatively, reduced PARP activity may be influenced by angiogenic *milieu* seen in microangiopathy conditions. For example, insulin-like growth factor-I (IGF-I) has been shown to promote angiogenesis by enhancing vascular endothelial growth factor (VEGF) expression via suppression of PARP activity (Beckert et al., 2006). However, these causality relationships need to be tested in larger samples and in prospective studies. Nevertheless, an increased oxidative DNA damage and PARP activity seen in our study in diabetic patients prior to the onset of vascular pathology is an important observation. Since PARP activation was also shown in healthy subjects at risk of developing diabetes as well as in established Type 2 diabetic patients (Szabo et al., 2002), these studies reveal opportunities for early therapeutic intervention. Moreover, increased DNA damage in our diabetic patients with microangiopathy compared to control subjects, emphasize a role for oxidative stress in the development and progression of microvascular complications. In fact, urinary 8-OHG has been reported as a biomarker of oxidative DNA damage in diabetic nephropathy patients (Hinokio et al., 1999; Wu, Chiou, Chang, & Wu, 2004; Xu et al., 2004) and higher serum 8-OHG levels were reported in diabetic patients with advanced microvascular complications (Shin et al., 2001). While decreased creatinine clearance is the earliest predictor of renal dysfunction, it is important to note in our study, that oxidative DNA damage was negatively correlated to creatinine clearance in diabetic patients. We also noted that the levels of oxidative DNA damage were significantly

Please cite this article in press as: Adaikalakoteswari, A., et al., Oxidative DNA damage and augmentation of poly(ADP-ribose) polymerase/nuclear factor-kappa B signaling in patients with Type 2 diabetes and microangiopathy, International Journal of Biochemistry and Cell Biology (2007), doi:10.1016/j.biocel.2007.04.013

($p = 0.019$) higher in patients with creatinine clearance of less than 70 ml/min compared with those with values greater than 70 ml/min. These observations warrant further studies so as to relate causality of oxidative DNA damage to the severity of diabetic microvascular complications.

The demonstration of oxidative DNA damage and augmentation of its downstream effectors in patients with Type 2 diabetes and microangiopathy in this work has several implications. Perhaps, this is one of the first population-based studies in which oxidative DNA damage was shown correlated to poor glycemic and lipid status on one hand and to augmentation of PARP/NFκB signals on the other hand. It is of interest to note that oxidative DNA damage in diabetes patients was not only correlated to HbA1c levels but also more significantly to post-prandial plasma glucose levels. While DCCT–EDIC outcomes (Hirsch & Brownlee, 2005) claim glycemic variability in addition to HbA1c as a risk factor for vascular complications, it has been recently shown that acute glucose fluctuations triggered more specific oxidative stress than sustained chronic hyperglycemia in patients with Type 2 diabetes (Monnier et al., 2006). In view of emerging evidence that oxidative DNA damage to be a biomarker in molecular epidemiological studies (Dalle-Donne, Rossi, Colombo, Giustarini, & Milzani, 2006; Heilbronn et al., 2006; Ilnytska et al., 2006), development of new strategies designed to detect DNA damage caused by oxidative stress may advance our understanding of the roles of such types of damage in the etiology of diabetes and its complications. In fact, a DNA adduct detection method (adductome approach) that uses liquid chromatography coupled with electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS) to detect multiple DNA adducts in human tissues as clinical biomarkers is also underway (Kanaly et al., 2006).

One of the limitations of this work is that being a cross-sectional study, a cause-and-effect relationship cannot be established. Nevertheless, it has provided a molecular evidence for genomic instability in patients with Type 2 diabetes and microangiopathy in Asian Indians. Asian Indians with Type 2 diabetes also exhibit decreased HDL (Misra, Luthra, & Vikram, 2004), hypoadiponectinemia (Mohan et al., 2005), hypoglutathionemia (Sampathkumar et al., 2005; Sampathkumar, Balasubramanyam, Tara, Rema, & Mohan, 2006), impaired antioxidant defense (Adaikalakoteswari et al., 2006), shortened telomeres (Adaikalakoteswari et al., 2005; Adaikalakoteswari, Balasubramanyam, Ravikumar, Deepa, & Mohan, 2007) and increased premature mortality rates (Mohan et al., 2006) all of which represent a state of increased oxidative stress and genomic instability. In this context, our work demonstrating increased oxidative DNA damage in Type 2 diabetic patients warrants further studies on the molecular aspects of both nuclear and mtDNA alterations which could ultimately lead to novel drugs and newer therapeutic options.

Acknowledgements

This work was supported by research grants from the Department of Science and Technology (DST & DST-FIST), Indian Council of Medical Research (ICMR) and the Department of Biotechnology (DBT), Government of India, New Delhi, India. AA acknowledges the financial assistance (Senior Research Fellowship) from the Council of Scientific & Industrial Research (CSIR), New Delhi, India. This is paper no. 50 from the Chennai Urban Rural Epidemiology Study (CURES).

References

Adaikalakoteswari, A., Balasubramanyam, M., & Mohan, V. (2005). Telomere shortening occurs in Asian Indian Type 2 diabetic patients. *Diabet. Med.*, *22*, 1151–1156.

Adaikalakoteswari, A., Balasubramanyam, M., Rema, M., & Mohan, V. (2006). Differential gene expression of NADPH oxidase (p22) and hemoxygenase-1 in patients with Type 2 diabetes and microangiopathy. *Diabet. Med.*, *23*, 666–674.

Adaikalakoteswari, A., Balasubramanyam, M., Ravikumar, R., Deepa, R., & Mohan, V. (2007). Association of telomere shortening with impaired glucose tolerance and diabetic macroangiopathy. *Atherosclerosis*, in press.

Balasubramanyam, M., Kimura, M., Aviv, A., & Gardner, J. P. (1993). Kinetics of calcium transport across the lymphocyte plasma membrane. *Am. J. Physiol.*, *265*, C321–C327.

Balasubramanyam, M. (2006). Antioxidants and cardiovascular disease—Where do we stand? *Asian J. Diab.*, *8*, 48–52.

Beckert, S., Farrahi, F., Perveen Ghani, Q., Aslam, R., Scheuenstuhl, H., Coerper, S., et al. (2006). IGF-I-induced VEGF expression in HUVEC involves phosphorylation and inhibition of poly(ADP-ribose)polymerase. *Biochem. Biophys. Res. Commun.*, *341*, 67–72.

Brownlee, M. (2001). Biochemistry and molecular cell biology of diabetic complications. *Nature*, *414*, 813–820.

Ceriello, A. (2003). New insights on oxidative stress and diabetic complications may lead to a "causal" antioxidant therapy. *Diabetes Care*, *26*, 1589–1596.

Chang, W. J., & Alvarez-Gonzalez, R. (2001). The sequence-specific DNA binding of NF-kappa B is reversibly regulated by the automodification reaction of poly (ADP-ribose) polymerase-1. *J. Biol. Chem.*, *276*, 47664–47670.

Choi, S. W., Benzie, I. F., Lam, C. S., Chat, S., Lam, J., Yiu, C. H., et al. (2005). Inter-relationships between DNA damage, ascorbic acid and glycaemic control in Type 2 diabetes mellitus. *Diabet. Med.*, *22*, 1347–1353.

Cohen-Armon, M., Visochek, L., Rozensal, D., Kalal, A., Geistrikh, I., Klein, R., et al. (2007). DNA-independent PARP-1 activation

by phosphorylated ERK2 increases Elk1 activity: A link to histone acetylation. *Mol. Cell*, *25*, 297–308.

Collins, A. R. (2004). The comet assay for DNA damage and repair: Principles, applications, and limitations. *Mol. Biotechnol.*, *26*, 249–261.

Collins, A., Duthie, S., & Dobson, V. (1993). Direct enzymatic detection of endogenous oxidative base damage in human lymphocyte DNA. *Carcinogenesis*, *14*, 1733–1735.

Dalle-Donne, I., Rossi, R., Colombo, R., Giustarini, D., & Milzani, A. (2006). Biomarkers of oxidative damage in human disease. *Clin. Chem.*, *52*, 601–623.

Deepa, M., Pradeepa, R., Rema, M., et al. (2003). The Chennai Urban Rural Epidemiology Study (CURES) study design and methodology (urban component) (CURES-I). *J. Assoc. Phys. India*, *51*, 863–870.

Demirbag, R., Yilmaz, R., & Kocyigit, A. (2005). Relationship between DNA damage, total antioxidant capacity and coronary artery disease. *Mutat. Res.*, *570*, 197–203.

Dincer, Y., Akcay, T., Alademir, Z., & Ilkova, H. (2003). Assessment of DNA base oxidation and glutathione level in patients with type 2 diabetes. *Mutat. Res.*, *9*(525), 129–130.

Evans, J. L., Goldfine, I. D., Maddux, B. A., & Grodsky, G. M. (2002). Oxidative stress and stress-activated signaling pathways: A unifying hypothesis of type 2 diabetes. *Endocr. Rev.*, *23*, 599–622.

Hassa, P. O., & Hottiger, M. O. (2002). The functional role of poly(ADP-ribose)polymerase 1 as novel coactivator of NF-kappaB in inflammatory disorders. *Cell. Mol. Life Sci.*, *59*, 1534–1553.

Heilbronn, L. K., et al. (2006). Effect of 6-month calorie restriction on biomarkers of longevity, metabolic adaptation, and oxidative stress in overweight individuals: A randomized controlled trial. *JAMA*, *295*, 1539–1548.

Hinokio, Y., Suzuki, S., Hirai, M., Chiba, M., Hirai, A., & Toyota, T. (1999). Oxidative DNA damage in diabetes mellitus: Its association with diabetic complications. *Diabetologia*, *42*, 995–998.

Hirsch, I. B., & Brownlee, M. (2005). Should minimal blood glucose variability become the gold standard of glycemic control? *J. Diabet. Complications*, *19*, 178–181.

Hofmann, M. A., Schiekofer, S., Kanitz, M., Klevesath, M. S., Joswig, M., Lee, V., et al. (1998). Insufficient glycemic control increases nuclear factor-kappa B binding activity in peripheral blood mononuclear cells isolated from patients with type 1 diabetes. *Diabetes Care*, *21*, 1310–1316.

Ilnytska, O., Lyzogubov, V. V., Stevens, M. J., Drel, V. R., Mashtalir, N., Pacher, P., et al. (2006). Poly(ADP-ribose) polymerase inhibition alleviates experimental diabetic sensory neuropathy. *Diabetes*, *55*, 1686–1694.

Irani, K. (2000). Oxidant signaling in vascular cell growth, death, and survival: A review of the roles of reactive oxygen species in smooth muscle and endothelial cell mitogenic and apoptotic signaling. *Circ. Res.*, *87*, 179–183.

Kabe, Y., Ando, K., Hirao, S., Yoshida, M., & Handa, H. (2005). Redox regulation of NF-kappaB activation: Distinct redox regulation between the cytoplasm and the nucleus. *Antioxid. Redox Signal.*, *7*, 395–403.

Kanaly, R. A., Hanaoka, T., Sugimura, H., Toda, H., Matsui, S., & Matsuda, T. (2006). Development of the adductome approach to detect DNA damage in humans. *Antioxid. Redox Signal.*, *8*, 993–1001.

Kunsch, C., & Medford, R. M. (1999). Oxidative stress as a regulator of gene expression in the vasculature. *Circ. Res.*, *85*, 753–766.

Larsen, L., Storling, J., Darville, M., Eizirik, D. L., Bonny, C., Billestrup, N., et al. (2005). Extracellular signal-regulated kinase is essential for interleukin-1-induced and nuclear factor kappaB-mediated gene expression in insulin-producing INS-1E cells. *Diabetologia*, *48*, 2582–2590.

Levine, R. L., Garland, D., Oliver, C. N., Amici, A., Climent, I., Lenz, A. G., et al. (1990). Determination of carbonyl content in oxidatively modified proteins. *Meth. Enzymol.*, *186*, 464–478.

Li, F., Szabo, C., Pacher, P., Southan, G. J., Abatan, O. I., Charniauskaya, T., et al. (2004). Evaluation of orally active poly(ADP-ribose) polymerase inhibitor in streptozotocin-diabetic rat model of early peripheral neuropathy. *Diabetologia*, *47*, 710–717.

Lin, T. K., Chen, S. D., Wang, P. W., Wei, Y. H., Lee, C. F., Chen, T. L., et al. (2005). Increased oxidative damage with altered antioxidative status in Type 2 diabetic patients harboring the 16189 T to C variant of mitochondrial DNA. *Ann. NY Acad. Sci.*, *1042*, 64–69.

Martin-Ventura, J. L., Blanco-Colio, L. M., Gomez-Hernandez, A., Munoz-Garcia, B., Vega, M., Serrano, J., et al. (2005). Intensive treatment with atorvastatin reduces inflammation in mononuclear cells and human atherosclerotic lesions in one month. *Stroke*, *36*, 1796–1800.

Misra, A., Luthra, K., & Vikram, N. K. (2004). Dyslipidemia in Asian Indians: Determinants and significance. *J. Assoc. Phys. India*, *52*, 137–142.

Mohan, V., Deepa, R., Pradeepa, R., Vimaleswaran, K. S., Mohan, A., Velmurugan, K., et al. (2005). Association of low adiponectin levels with the metabolic syndrome—The Chennai Urban Rural Epidemiology Study (CURES-4). *Metabolism*, *54*, 476–481.

Mohan, V., Meera, R., Premalatha, G., Deepa, R., Miranda, P., & Rema, M. (2000). Frequency of proteinuria in type 2 diabetes mellitus seen at a diabetes centre in southern India. *Postgrad. Med. J.*, *76*, 569–573.

Mohan, V., Ravikumar, R., ShanthiRani, S., & Deepa, R. (1998). Intimal medial thickness of the carotid artery in south Indian diabetic and nondiabetic subjects: The Chennai Urban Population Study (CUPS). *Diabetologia*, *433*, 494–499.

Mohan, V., Shanthirani, C. S., Deepa, M., Deepa, R., Unnikrishnan, R. I., & Datta, M. (2006). Mortality rates due to diabetes in a selected urban South Indian Population—The Chennai Urban Population Study (CUPS-16). *JAPI*, *54*, 113–117.

Monnier, L., Mas, E., Ginet, C., Michel, F., Villon, L., Cristol, J. P., & Colette, C. (2006). Activation of oxidative stress by acute glucose fluctuations compared with sustained chronic hyperglycemia in patients with type 2 diabetes. *JAMA*, *295*, 1681–1687.

Morrow, J. D. (2005). Quantification of isoprostanes as indices of oxidant stress and the risk of atherosclerosis in humans. *Arterioscler. Thromb. Vasc. Biol.*, *25*, 279–286.

Ohga, S., Shikata, K., Yozai, K., Okada, S., Ogawa, D., Usui, H., et al. (2007). Thiazolidinedione ameliorates renal injury in experimental diabetic rats through anti-inflammatory effects mediated by inhibition of NF-{kappa}B activation. *Am. J. Physiol.*, *292*, F1141–F1150.

Pacher, P., Liaudet, L., Soriano, F. G., Mabley, J. G., Szabo, E., & Szabo, C. (2002). The role of poly(ADP-ribose) polymerase in the development of myocardial and endothelial dysfunction in diabetes mellitus. *Diabetes*, *51*, 514–521.

Pacher, P., Obrosova, I. G., Mabley, J. G., & Szabo, C. (2005). Role of nitrosative stress and peroxynitrite in the pathogenesis of diabetic complications. Emerging new therapeutical strategies. *Curr. Med. Chem.*, *12*, 267–275.

Pitozzi, V., Giovannelli, L., Bardini, G., Rotella, C. M., & Dolara, P. (2003). Oxidative DNA damage in peripheral blood cells in type 2 diabetes mellitus: Higher vulnerability of polymorphonuclear leukocytes. *Mutat. Res.*, *529*, 129–133.

Please cite this article in press as: Adaikalakoteswari, A., et al., Oxidative DNA damage and augmentation of poly(ADP-ribose) polymerase/nuclear factor-kappa B signaling in patients with Type 2 diabetes and microangiopathy, International Journal of Biochemistry and Cell Biology (2007), doi:10.1016/j.biocel.2007.04.013

Rema, M., Mohan, V., Deepa, R., & Ravikumar, R. (2004). Association of carotid intimal medial thickness and arterial stiffness with diabetic retinopathy—The Chennai Urban Rural Epidemiology Study [CURES-2]. *Diabetes Care*, *27*, 1962–1967.

Sampathkumar, R., Balasubramanyam, M., Sudarslal, S., Rema, M., Mohan, V., & Balaram, P. (2005). Increased glutathionylated hemoglobin (HbSSG) in type 2 diabetes subjects with microangiopathy. *Clin. Biochem.*, *38*, 892–899.

Sampathkumar, R., Balasubramanyam, M., Tara, C., Rema, M., & Mohan, V. (2006). Association of hypoglutathionemia with reduced Na+/K+ ATPase activity in type 2 diabetes and microangiopathy. *Mol. Cell. Biochem.*, *282*, 169–176.

Sampson, M. J., Winterbone, M. S., Hughes, J. C., Dozio, N., & Hughes, D. A. (2006). Monocyte telomere shortening and oxidative DNA damage in type 2 diabetes. *Diabetes Care*, *29*, 283–289.

Schmeisser, A., Soehnlein, O., Illmer, T., Lorenz, H. M., Eskafi, S., Roerick, O., et al. (2004). ACE inhibition lowers angiotensin II-induced chemokine expression by reduction of NF-kappaB activity and AT1 receptor expression. *Biochem. Biophys. Res. Commun.*, *325*, 532–540.

Shall, S., & de Murcia, G. (2000). Poly(ADP-ribose) polymerase-1: What have we learned from the deficient mouse model? *Mutat. Res.*, *460*, 1–15.

Shin, M. J., Cho, E. Y., Jang, Y., Lee, J. H., Shim, W. H., Cho, S. Y., et al. (2005). A beneficial effect of simvastatin on DNA damage in 242T allele of the NADPH oxidase p22phox in hypercholesterolemic patients. *Clin. Chim. Acta*, *360*, 46–51.

Shin, C. S., Moon, B. S., Park, K. S., Kim, S. Y., Park, S. J., Chung, M. H., et al. (2001). Serum 8-hydroxy-guanine levels are increased in diabetic patients. *Diabetes Care*, *24*, 733–737.

Singh, N., McCoy, M., Tice, R., & Schneider, L. (1988). A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp. Cell Res.*, *175*, 84–191.

Speit, G., Schutz, P., Bonzheim, I., Trenz, K., & Hoffmann, H. (2004). Sensitivity of the FPG protein towards alkylation damage in the comet assay. *Toxicol. Lett.*, *146*, 151–158.

Szabo, C., Zanchi, A., Komjati, K., Pacher, P., Krolewski, A. S., Quist, W. C., et al. (2002). Poly(ADP-ribose) polymerase is activated in subjects at risk of developing type 2 diabetes and is associated with impaired vascular reactivity. *Circulation*, *106*, 2680–2686.

Varghese, A., Deepa, R., Rema, M., & Mohan, V. (2001). Prevalence of microalbuminuria in type 2 diabetes mellitus at a diabetes centre in southern India. *Postgrad. Med. J.*, *77*, 399–402.

Wahlberg, G., Carlson, L. A., Wasserman, J., & Ljungqvist, A. (1985). Protective effect of nicotinamide against nephropathy in diabetic rats. *Diab. Res.*, *2*, 307–312.

Wu, L. L., Chiou, C. C., Chang, P. Y., & Wu, J. T. (2004). Urinary 8-OHdG: A marker of oxidative stress to DNA and a risk factor for cancer, atherosclerosis and diabetics. *Clin. Chim. Acta*, *339*, 1–9.

Xu, G. W., Yao, Q. H., Weng, Q. F., Su, B. L., Zhang, X., & Xiong, J. H. (2004). Study of urinary 8-hydroxydeoxyguanosine as a biomarker of oxidative DNA damage in diabetic nephropathy patients. *J. Pharm. Biomed. Anal.*, *36*, 101–104.

Yagi, K. (1976). A simple fluorometric assay for lipoperoxide in blood plasma. *Biochem. Med.*, *15*, 212–216.

Zheng, L., Szabo, C., & Kern, T. S. (2004). Poly(ADP-ribose) polymerase is involved in the development of diabetic retinopathy via regulation of nuclear factor-kappaB. *Diabetes*, *53*, 2960–2967.

Please cite this article in press as: Adaikalakoteswari, A., et al., Oxidative DNA damage and augmentation of poly(ADP-ribose) polymerase/nuclear factor-kappa B signaling in patients with Type 2 diabetes and microangiopathy, International Journal of Biochemistry and Cell Biology (2007), doi:10.1016/j.biocel.2007.04.013