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Differential gene expression of NADPH oxidase ($p22^{phox}$) and hemoxygenase-1 in patients with Type 2 diabetes and microangiopathy

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Abstract

Aims While the downstream effects of increased reactive oxygen species (ROS) in the pathogenesis of diabetes were well studied, only a few studies have explored the cellular sources of ROS. We examined whether protection against oxidative stress is altered in patients with diabetes and microangiopathy by examining changes in NADPH oxidase ($p22^{phox}$) and hemoxygenase-1 (HO-1) levels.

Methods NADPH oxidase ($p22^{phox}$) and HO-1 gene expression were probed by RT-PCR using leucocytes from patients with Type 2 diabetes without (n = 19) and with microangiopathy (n = 20) and non-diabetic subjects (n = 17). Levels of lipid peroxidation as measured by thiobarbituric reactive substances (TBARS) and protein carbonyl content (PCO) were determined by fluorimetric and spectrophotometric methods, respectively.

Results $p22^{phox}$ gene expression (mean ± SE) was significantly ($P < 0.05$) higher in diabetic patients with (0.99 ± 0.04) and without microangiopathy (0.86 ± 0.05) compared with control subjects (0.66 ± 0.05). Consistent with the mRNA data, the $p22^{phox}$ protein expression and NADPH oxidase activity was also increased in cells from diabetic patients compared with control subjects. However, HO-1 gene expression was significantly ($P < 0.05$) lower in patients with (0.73 ± 0.03) and without microangiopathy (0.85 ± 0.02) compared with control subjects (1.06 ± 0.03). The mean (± SE) levels of TBARS were significantly ($P < 0.05$) higher in diabetic patients with (14.36 ± 1.3 nM/ml) and without microangiopathy (12.20 ± 1.3 nM/ml) compared with control subjects (8.58 ± 0.7 nM/ml). The protein carbonyl content was also significantly ($P < 0.05$) higher in diabetic patients with (1.02 ± 0.04 nmol/mg protein) and without microangiopathy (0.84 ± 0.06 nmol/mg protein) compared with control subjects (0.48 ± 0.02 nmol/mg protein). In diabetic subjects, increased $p22^{phox}$ gene expression was negatively correlated with HO-1 and positively correlated with TBARS, PCO, HbA_{1c} and diabetes duration. In contrast, HO-1 gene expression was correlated negatively with p22phox, TBARS, PCO, HbA_{1c} and diabetes duration.

Conclusion Our results indicate that increased oxidative damage is seen in Asian Indians with Type 2 diabetes and microangiopathy and is associated with increased NADPH oxidase ($p22^{phox}$) and decreased HO-1 gene expression.

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Keywords Asian Indians, diabetes mellitus, hemoxygenase-1, NADPH oxidase, oxidative stress

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Abbreviations DNPH, 2, 4-dinitrophenylhydrazine; DPI, diphenylene iodonium; HDL, high-density lipoprotein; HO-1, hemoxygenase-1; LDL, low-density lipoprotein; PBS, phosphate-buffered saline; PCO, protein carbonyl content; PMA, phorbol myristate acetate; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; TBARS, thiobarbituric reactive substances

Introduction

There is growing evidence linking the pathogenesis of diabetes mellitus with oxidative stress [1,2]. Because oxidative stress results from increased production or decreased removal of reactive oxygen species (ROS), many studies are now targeted at identifying the cellular sources of ROS. While there are multiple sources of ROS, notably xanthine oxidase, uncoupled nitric oxide synthase, and mitochondria, a major role of NADPH oxidase as a source of superoxide and hydrogen peroxide has recently been emphasized [3]. Although NADPH oxidase was identified and characterized primarily in phagocytic leucocytes [4], its presence and specific signalling roles have been implicated in many cell types such as fibroblasts, endothelial cells, vascular smooth muscle cells, adipocytes and pancreatic B-cells [5–7]. Hence, there is considerable interest in studying whether NADPH oxidase activity contributes to the pathogenesis or progression of micro- and macrovascular complications of diabetes.

It is also important to know how the expression of genes associated with cellular defence against oxidative stress is altered in patients with Type 2 diabetes. Hemoxygenases (HOs) catalyse the rate-limiting step in heme degradation, resulting in the formation of iron, carbon monoxide, and biliverdin, the latter being subsequently converted to bilirubin by biliverdin reductase. Recent attention has focused on the biological effects of product(s) of this enzymatic reaction, which have important antioxidant, anti-inflammatory, and cytoprotective functions [8]. Although induction of HO is expected to be an adaptive and beneficial response to various stimuli, including oxidative stress, the functional significance of HO gene products is tightly regulated by the metabolic milieu and, hence, their alterations need to be studied in diabetes.

Thus, we quantified NADPH oxidase (subunit $p22^{phox}$) and hemoxygenase-1 (HO-1) gene expression in patients with Type 2 diabetes with and without microangiopathy, along with markers of oxidative stress such as lipid peroxidation and protein carbonyls. As target-specific cellular profiling in humans is difficult to ascertain *in vivo*, we used human leucocytes, a readily accessible cell model [9,10].

Subjects and methods

Sample selection

Study subjects were recruited from the Chennai Urban Rural Epidemiology Study (CURES), which is an ongoing epidemiological study, conducted on a representative population of Chennai. The methodology of the study has been published elsewhere [11,12]. Details such as age, sex and, in diabetic subjects, duration of diabetes and other details of diabetic therapy were recorded and clinical examination was carried out in all subjects. All non-diabetic subjects underwent oral glucose tolerance tests using a 75-g glucose load. Those who were confirmed by oral glucose tolerance test to have fasting plasma glucose < 6.1 mmol/l and a 2-h plasma glucose value < 7.8 mmol/l were categorized as normal glucose tolerance. For the present study, we randomly selected (using computer-generated random numbers) 20 diabetic subjects with microangiopathy, 19 diabetic subjects without any microangiopathy and 17 subjects with normal glucose tolerance.

Microangiopathy was diagnosed if nephropathy and/or retinopathy were present. Nephropathy was defined as either persistent proteinuria (≥ 500 mg/day) or persistent microalbuminuria [if albuminuria estimated by albumin creatinine ratio exceeded 30 mg/g of creatinine] in the absence of urinary tract infection [13,14]. Retinopathy was assessed as described earlier [12,15] using fundus photography. 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 colour retinal photography with a Zeiss FF 450 plus camera using 35-mm colour transparencies. The photographs were graded against standard photographs of the Early Treatment Diabetic Retinopathy Study grading system for severity of retinopathy. Hypertension was diagnosed if the subjects had been treated with anti-hypertensive drugs or had systolic blood pressure ≥ 140 mmHg or diastolic blood pressure ≥ 90 mmHg. Diabetic subjects without 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 mg/g creatinine). They also had no history of angina or myocardial infarction and normal 12-lead resting electrocardiogram. Informed consent was obtained from all study subjects and the institutional ethics committee approved the study.

Physical examination included height, weight, and 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 stored at –70°C until the assays were performed. Biochemical analyses were carried out on a Hitachi-912 Autoanalyser (Hitachi, Mannheim, Germany) using kits supplied by Roche Diagnostics (Mannheim, Germany). Fasting plasma glucose (GOD-POD (glucose oxidase peroxidase) method), serum cholesterol (CHOD-PAP (cholesterol oxidase peroxidase) method), serum triglycerides (GPO-PAP (glycerol phosphate oxidase peroxidase) method) and high-density lipoprotein (HDL) cholesterol (direct method–polyethylene glycol-pretreated enzymes) were measured. Low-density lipoprotein (LDL) cholesterol was calculated using the Friedewald formula [16].

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Glycated haemoglobin (HbA_{1c}) was estimated by HPLC using the Variant machine (Bio-Rad Laboratories, Hercules, CA, USA).

RNA extraction

Total RNA from leucocytes (~5×10^6) was extracted using Trizol reagent (Sigma, St Louis, MO, USA). 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 optical density ratio between 1.8 and 2.0.

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, Valencia, CA, USA), 200 units murine leukaemia virus reverse transcriptase (Amersham Pharamacia Biotech, Piscataway, NJ, USA), 2.5 mm dNTPs and 50 units ribonuclease inhibitor in a Thermocycler (Biorad, Hercules, CA, USA) (55 min at 37°C, 5 min at 95°C) [10].

The $p22^{phox}$ mRNA expression PCR was probed using specific primers; their sequence is 5′-GTTTGTGTGCCTGCTGGAGT-3′ and 5′-TGGGCGGCTGCTTGATGGT-3′ (nucleotide positions 168–187 and 465–485, respectively). The conditions of amplification were: 95°C for 1 min, 62°C for 1 min and 72°C for 1 min for 30 cycles of amplification. The number of cycles was determined to assure that the amplification occurs in the exponential phase. The oligomer primers used for HO-1 gene expression were 5′-CAGGCAGAGAATGCTGAGTTC-3′ and 5′-GCTTCACATAGCGCTGCA-3′ (nucleotide positions 79–99 and 332–349, respectively). The conditions of amplification were: 94°C for 30 s, 58°C for 1 min and 72°C for 1 min for 26 cycles of amplification. PCR products were separated by 2% agarose gel electrophoresis. The housekeeping GAPDH PCR products obtained by amplifying primers were used as an internal control.

Evaluation of $p22^{phox}$ and the HO-1 gene product

$p22^{phox}$ and HO-1 gene expression were quantified using Bio-Rad gel documentation and semiquantitative analysis using its software. The ratio of $p22^{phox}$ and HO-1 to GAPDH PCR product, expressed as peak density, was used as indices of $p22^{phox}$ and HO-1 gene expression (in densitometric units).

Isolation of lymphocytes

Freshly collected peripheral blood was carefully layered on histopaque gradient and centrifuged at 1600 r.p.m. (500 *g*) for 30 min. The buffy-coat interface representing lymphocytes was aspirated and washed three times in phosphate-buffered saline (PBS) pH 7.4. Protein analysis and ROS measurements were done in a subset of subjects ($n = 10$ in each category).

Western blot analysis

Protein extracts were obtained from lymphocytes isolated from 5 ml peripheral blood by lysis of cells in RIPA buffer [50 mM Tris pH 8.0, 150 mM NaCl, 0.1% sodium dodecyl sulfate (SDS), 0.02% sodium azide, 1% NP40, 0.5% sodium deoxycholate]. The cell extract was centrifuged (5 min, 10 000 *g*) and the supernatant was stored at −80°C. Protein concentration of the samples was determined using Bradford's assay. Prior to analysis, 50 μg protein was boiled for 5 min in sample buffer [20 mM Tris-HCl pH 6.8, 1% dithiothreitol, 1% SDS, 20% glycerol, 0.1% bromophenol blue]. The proteins were separated by 12% SDS–polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane, and incubated for 3 h in PBS-0.1% Tween 20 buffer containing 5% bovine serum albumin. The membrane was then incubated with rabbit anti-$p22^{phox}$ antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1 : 200 in blocking solution for 1 h at room temperature. The membrane was washed extensively with PBS-0.1% Tween 20 buffer and incubated with goat anti-rabbit IgG-alkaline phosphatase conjugated antibody for 1 h at room temperature. The membrane was washed again and membrane-bound antibodies were visualized by BCIP/NBT substrate (Bangalore Genei, Bangalore, India). Immunoblotting with β-actin was performed as loading control.

Quantification of $p22^{phox}$ protein expression

$p22^{phox}$ protein expression was determined semiquantitatively using Bio-Rad gel documentation software. The ratio of $p22^{phox}$ to β-actin western blot products, expressed as peak density, was used as indices of $p22^{phox}$ expression (in densitometric units).

Measurement of intracellular ROS generation and NADPH oxidase activity

To measure intracellular ROS production, cells were loaded with 10 μM 2′,7′-dichlorofluorescein diacetate (H_2DCF-DA) for 45 min at room temperature. Cells were centrifuged to remove the extracellular dye, suspended in HEPES buffer, added to microplate wells and challenged with 500 μM phorbol myristate acetate (PMA). ROS generation was monitored in Fluoromax-3 spectrofluorimeter (excitation set at 485 nm and emission at 530 nm) as a change in the fluorescence intensity because of the conversion of non-fluorescent dichlorofluorescein diacetate to the highly fluorescent compound, 2′,7′-dichlorofluorescein in the cells [17]. In order to estimate the relative NADPH oxidase activity, cells treated with PMA were incubated with different concentrations (0.1, 0.5, 1.0, 5.0, 10.0 μM) of diphenylene iodonium (DPI), an inhibitor of NADPH oxidase. The DPI concentration (IC_{50}) required to inhibit 50% of NADPH activity was calculated by a curve fitting program (BioDataFit 1.02; Chang Broscience Inc, Castro Valley, CA, USA).

Lipid peroxidation

Plasma levels of malonodialdehyde, a marker of lipid peroxidation was measured as thiobarbituric acid reactive substances (TBARS) by fluorescence methodology [18,19]. Briefly, plasma (200 μl) was treated with 8.1% SDS and 20% acetic acid to solubilize protein and precipitate it and then heated with thiobarbituric acid for 1 h at 95°C. The supernatant was then extracted with butanol : pyridine (15 : 1) to yield a fluorescent product, which was detected by excitation at 535 nm and emission at 553 nm.

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Table 1 Clinical characteristics of the study subjects

Parameters	Control (n = 17)	Type 2 diabetes without microangiopathy (n = 19)	Type 2 diabetes with microangiopathy (n = 20)
Age (years)	47 ± 7	53 ± 9*	55 ± 7*
Duration of diabetes (years)	0	5 ± 3	15 ± 5†
Fasting plasma glucose (mmol/l)	4.7 ± 0.3	7.7 ± 1.6*	9.1 ± 3.1*
Glycated haemoglobin (%)	5.5 ± 0.4	7.4 ± 1.6*	8.9 ± 1.6*
Systolic blood pressure (mmHg)	110 ± 13	134 ± 17*	135 ± 18*
Diastolic blood pressure (mmHg)	70 ± 6	79 ± 8*	78 ± 6*
Serum cholesterol (mmol/l)	3.9 ± 0.3	5.0 ± 0.8*	5.4 ± 0.8*
Serum triglycerides (mmol/l)	1.1 ± 0.4	1.8 ± 0.7*	1.9 ± 0.8*
Serum HDL cholesterol (mmol/l)	1.2 ± 0.2	1.1 ± 0.2*	0.9 ± 0.2*
Serum LDL cholesterol (mmol/l)	2.5 ± 0.3	3.2 ± 0.9*	3.4 ± 0.9*
Cholesterol : HDL ratio	3.2 ± 0.6	5.0 ± 1.4*	5.0 ± 0.9*

*$P < 0.05$ compared with control; †$P < 0.05$ compared with diabetes without microangiopathy.

Absolute malonodialdehyde levels were calculated using the regression parameters obtained using various concentrations (0.25–5.0 nM) of the standard, 1,1′,3,3′,-tetramethoxypropane. Inter- and intra-assay coefficients of variation of the above method were < 5 and 10%, respectively.

Protein carbonyls

Carbonyl content was quantified by the 2, 4-dinitrophenylhydrazine (DNPH) assay with slight modifications [20]. To 200 µl plasma, 800 µl of 10 mM DNPH in 2 M HCl was added and allowed to stand at room temperature for 1 h, vortexing every 10–15 min to facilitate the reaction with proteins. Plasma protein was precipitated with 20% trichloroacetic acid (1 : 1 v/v ratio) and centrifuged at 4°C, 10 000 *g* for 5 min. Clear supernatant was discarded and the pellet was washed three times with 1 ml of ethanol : ethylacetate (1 : 1 v/v ratio). Finally, pellets were dissolved in 1 ml of 6M-guanidine hydrochloride at 37°C. After centrifugation at 10 000 *g* for 5 min to precipitate insoluble material, the samples were read against complementary blank on the maximum absorbance showed at 365 nm. Blanks were run with 2 M HCl alone instead of DNPH reagent. All measurements were carried out in duplicate. The intra- and interassay coefficients of variation were 2.2 and 2.8%, respectively. Carbonyl content was expressed in nmol per mg of protein, using a molar absorption coefficient of 22 000/M/cm. Protein concentration was determined using a standard curve with bovine serum albumin (0.25–5.0 mg/ml) dissolved in guanidine hydrochloride and read at 280 nm.

Statistical analysis

Comparisons between groups were performed using one-way ANOVA and a *P*-value of less than 0.05 was considered statistically significant. Pearson correlation analysis was carried out between variables. Risk variables that had significant association with dependent variable ($p22^{phox}$ and HO-1 gene expression) on univariate regression, were included as independent variables in multiple linear regression analysis. Forward regression analysis was used to investigate relationships between the $p22^{phox}$ and HO-1 gene expression and age, HbA_{1c}, HDL, HO-1 gene expression, TBARS and protein carbonyl content (PCO). Care was taken to avoid intercorrelated variables in the regression equation. An IC_{50} calculation was performed using a curvefit program (BioDataFit 1.02). All analysis was carried out using Windows-based SPSS statistical package (Version 10.0, SAS Institute, Chicago, IL, USA).

Results

Table 1 shows the characteristics of the study subjects. None of the diabetic patients had ketonuria or a history of diabetic ketosis. Of the 39 Type 2 diabetic subjects, 65% took oral glucose lowering agents (OHA) and OHA plus insulin was taken by others (35%). Six of the diabetic subjects without microangiopathy and 10 with microangiopathy, were on ACE inhibitors and/or statin or aspirin therapy, in addition to glucose-lowering agents. Diabetic patients with and without microangiopathy had significantly higher fasting plasma glucose, HbA_{1c}, serum cholesterol and triglycerides compared with control subjects.

Figure 1(a) is a representative illustration of the gene expression patterns of $p22^{phox}$, HO-1 and GAPDH in the study groups. As seen in Fig. 1(b), age-adjusted $p22^{phox}$ gene expression (mean ± SE) was significantly ($P < 0.05$) higher in diabetic patients with (0.99 ± 0.04) and without microangiopathy (0.86 ± 0.05) compared with control subjects (0.66 ± 0.05). Conversely, the age-adjusted HO-1 gene expression was significantly ($P < 0.05$) lower in patients with (0.73 ± 0.03) and without microangiopathy (0.85 ± 0.02) compared with control subjects (1.06 ± 0.03) (Fig. 1c).

Consistent with the mRNA data, $p22^{phox}$ protein expression (mean ± SE) in diabetes subjects with (0.77 ± 0.05) and without (0.67 ± 0.06) microangiopathy was significantly higher when compared with control (0.44 ± 0.06) subjects (Fig. 2a and b). PMA-inducible ROS generation in lymphocytes (arbitrary fluorescence units mean ± SE) was also significantly increased in diabetic patients without (2221 ± 355) and with complications

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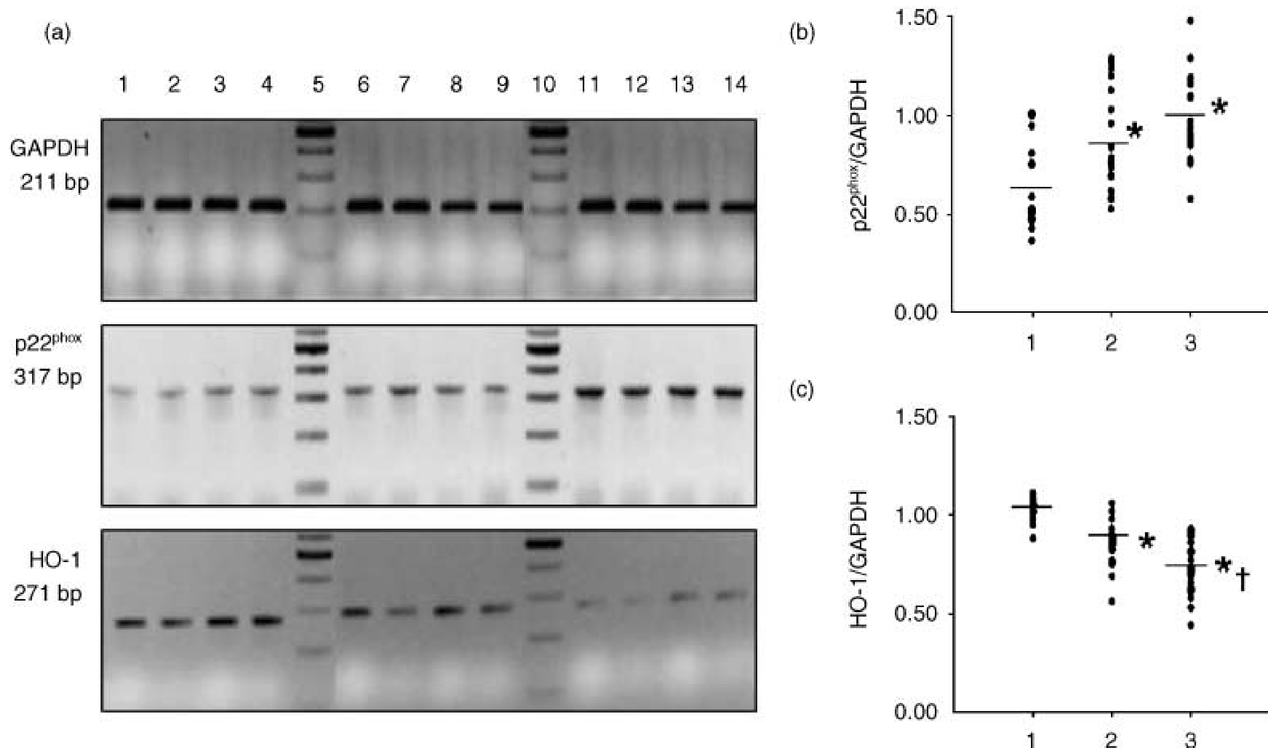

Figure 1 mRNA expression patterns in the study group. (a) Representative patterns of gene expression of p22^{phox}, hemoxygensase-1 and GAPDH in control subjects (lanes 1–4), diabetic subjects without microangiopathy (lanes 6–9) and diabetic subjects with microangiopathy (lanes 11–14). Lanes 5 and 10 show the DNA ladder (100–500 bp). Densitometric analysis was carried out for p22^{phox} and hemoxygenase-1 gene expression in leucocytes from (i) control subjects (*n* = 17), (ii) diabetic subjects without microangiopathy (*n* = 19) and (iii) diabetic subjects with microangiopathy (*n* = 20). Expression of both p22^{phox} and HO-1 mRNA were adjusted for the expression of the housekeeping gene GAPDH. (b) Estimated p22^{phox} mRNA expression (mean ± SE, indicated by a horizontal line) for 1, 2 and 3 were 0.66 ± 0.05, 0.86 ± 0.05 and 0.99 ± 0.04, respectively. (c) Mean HO-1 mRNA expression for 1, 2 and 3 were 1.06 ± 0.03, 0.85 ± 0.02 and 0.73 ± 0.03, respectively. *$P < 0.05$ compared with control values, †$P < 0.05$ compared with values in diabetic subjects without microangiopathy.

(2325 ± 368) compared with control (880 ± 84) subjects (data not shown). When DPI, an inhibitor of NADPH oxidase, was used in a dose-dependent way to determine relative NADPH oxidase activity, the concentration of DPI to achieve 50% reduction in the PMA-inducible ROS was greater in diabetic patients with (3.26 μM) and without (2.77 μM) microangiopathy than in control subjects (1.32 μM) (Fig. 2c). Thus, NADPH oxidase activity was increased in cells from diabetic patients. p22^{phox} mRNA levels positively correlated with both protein ($r = 0.51$; $P = 0.02$) and NADPH oxidase activity ($r = 0.55$; $P = 0.01$) levels.

The levels of TBARS were significantly ($P < 0.05$) higher in patients with (14.36 ± 1.3 nM/ml) and without microangiopathy (12.20 ± 1.3 nM/ml) compared with control subjects (8.58 ± 0.7 nM/ml) (Fig. 3a). PCO levels were also significantly ($P < 0.05$) higher in patients with (1.02 ± 0.04 nmol/mg protein) and without microangiopathy (0.84 ± 0.06 nmol/mg protein) compared with control subjects (0.48 ± 0.02 nmol/mg protein) (Fig. 3b).

To determine whether ACE inhibitor/statin therapies interfere with cellular oxidative stress and/or expression of NADPH oxidase components, we analysed the data related to TBARS, PCO, p22^{phox} and HO-1 mRNA levels in diabetic patients by dividing them into two groups: those taking only glucose-lowering-agents (group A, *n* = 23) and those taking glucose-lowering agents along with ACE inhibitor/statin (group B, *n* = 16). No significant differences in estimated TBARS, PCO, p22^{phox} and HO-1 mRNA levels were observed (data not shown).

In diabetic subjects, increased p22^{phox} gene expression correlated negatively with HO-1 ($r = -0.31$, $P = 0.05$) and positively to TBARS ($r = 0.58$, $P = 0.0001$), PCO ($r = 0.52$, $P = 0.001$), HbA_{1c} ($r = 0.34$, $P = 0.03$) and diabetes duration ($r = 0.33$, $P = 0.04$). HO-1 gene expression correlated negatively with p22^{phox} ($r = -0.31$, $P = 0.05$), TBARS ($r = -0.32$, $P = 0.04$), PCO ($r = -0.32$, $P = 0.04$), HbA_{1c} ($r = -0.35$, $P = 0.03$) and diabetes duration ($r = -0.33$, $P = 0.04$). When all study subjects were analysed together, p22^{phox} gene expression also correlated negatively with HDL ($r = -0.3$; $P = 0.02$). In contrast, HO-1 gene expression correlated positively with HDL ($r = 0.34$; $P = 0.01$) and negatively with diastolic blood pressure ($r = -0.31$; $P = 0.02$) and systolic blood pressure ($r = -0.36$; $P = 0.007$).

When age, HbA_{1c}, HDL, HO-1 gene expression, TBARS and PCO were included as potential independent variables in the forward regression analysis, TBARS and PCO remained in the final regression equation as predictors of p22^{phox} gene

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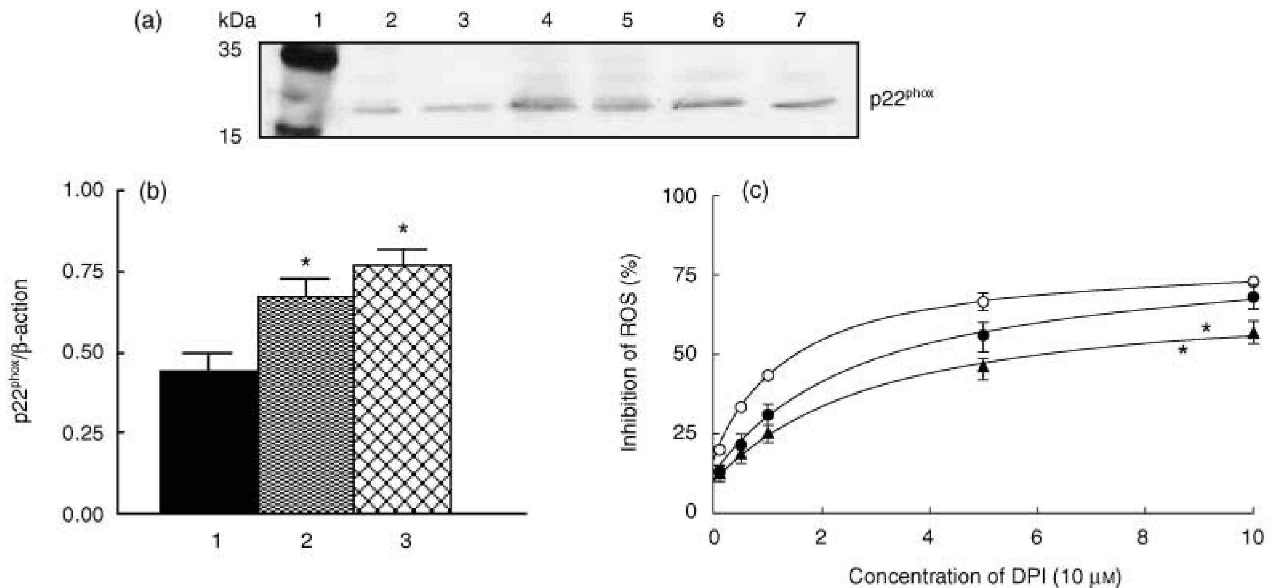

Figure 2 $p22^{phox}$ protein analysis and NADPH oxidase activity in the study subjects. (a) Representative illustration of protein expression pattern of $p22^{phox}$ in control subjects (lanes 2 and 3), diabetic subjects without microangiopathy (lanes 4 and 5) and diabetic subjects with microangiopathy (lanes 6 and 7). Lane 1 represents molecular weight marker (15–35 kDa). (b) Cumulative data after densitometric analysis for $p22^{phox}$ protein expression in lymphocytes from (1) control subjects (n = 10), (2) diabetic subjects without microangiopathy (n = 10) and (3) diabetic subjects with microangiopathy (n = 10). Expression of $p22^{phox}$ was adjusted for the expression of the housekeeping gene β-actin. Estimated $p22^{phox}$ protein expression (mean ± SE) for (1), (2) and (3) was 0.44 ± 0.06, 0.67 ± 0.06 and 0.77 ± 0.05, respectively. *$P < 0.05$ compared with control values. (c) IC_{50} data for NADPH oxidase activity in the study groups. NADPH oxidase activity was inferred from diphenylene iodonium (DPI) inhibition of PMA-induced ROS generation in cells as detailed in the Subjects and methods. Estimated IC_{50} of DPI for control subjects (n = 10), diabetic subjects without microangiopathy (n = 10) and diabetic subjects with microangiopathy (n = 10) was 1.32 (○), 2.77 (●) and 3.26 μM (▲), respectively. *$P < 0.05$ compared with control values.

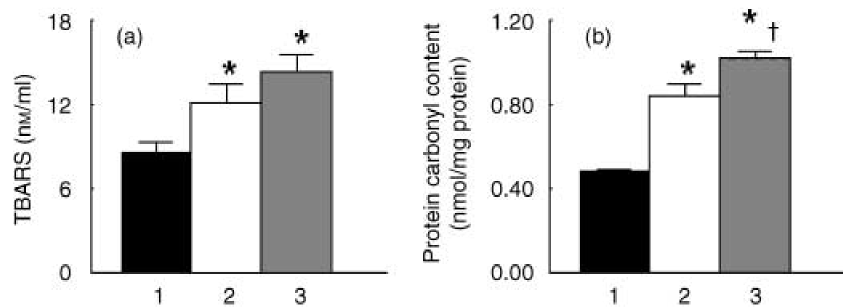

Figure 3 Plasma levels of lipid peroxidation (TBARS) and protein carbonyls in the study subjects: (1) control subjects (n = 17), (2) diabetic subjects without microangiopathy (n = 19) and (3) diabetic subjects with microangiopathy (n = 20). (a) Mean ± SE TBARS in samples (1), (2) and (3) were 8.58 ± 0.7, 12.2 ± 1.3 and 14.36 ± 1.3, respectively. (b) Mean ± SE protein carbonyl content for (1), (2) and (3) were 0.48 ± 0.02, 0.84 ± 0.06 and 1.02 ± 0.04, respectively. *$P < 0.05$ compared with control values, †$P < 0.05$ compared with values in diabetic subjects without microangiopathy.

expression (Table 2). In a similar analysis with HO-1 as the dependant variable, HbA_{1c} and PCO were significant predictors of HO-1 gene expression.

Discussion

Asian Indians have high prevalence rates of Type 2 diabetes and premature coronary heart disease [21]. A number of risk factors for these diseases may operate via pro-inflammatory and pro-oxidant mechanisms. Our earlier work indicated hyper-insulinemia [22], increased insulin resistance [23], increased pro-thrombogenic factors [24], decreased adiponectin [25], increased advanced glycation [26] and shortened telomeres [19] in Asian Indian patients with Type 2 diabetes. It is possible that these alterations could either induce oxidative damage or alternatively arise from increased oxidative stress. The present study is the first to our knowledge to report on the proximal molecular defects related to oxidative stress and oxidative damage in relation to diabetes and microangiopathy in Asian Indians. These observations are significant in that India has the largest number of people with diabetes in the world [27] and increased oxidative stress and inflammation could be one of the molecular mechanisms for predisposition to diabetes and its complications.

Increased NADPH oxidase activity is implicated in the pathogenesis of pancreatic B-cell apoptosis [7] and in the development

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Table 2 Multivariate regression analysis for the association of p22^{phox} and hemoxygenase-1 gene expression in Type 2 diabetic patients

	Unstandardized coefficients		
Model	β	se	Significance
p22^{phox}			
TBARS	0.014	0.005	0.009
PCO	0.241	0.098	0.017
Hemoxygenase-1			
HbA_{1c}	–0.031	0.010	0.003
PCO	–0.192	0.064	0.004

of diabetic complications, especially diabetic nephropathy [28]. This stimulated us to look at these parameters in Asian Indian diabetic subjects with and without microangiopathy, as they are believed to have a greater predisposition to diabetic nephropathy [29]. Our findings show that gene expression of NADPH subunit p22^{phox} is increased in leucocytes of Asian Indian Type 2 diabetic subjects, and more so in those with microangiopathy. Consistent with the mRNA data, the p22^{phox} protein expression and NADPH oxidase activity were also increased in cells from diabetic patients. These data are in agreement with those of Kim *et al.* [30] and Avogaro *et al.* [10]. In an experimental diabetic nephropathy study, Satoh *et al.* [31] have observed NADPH oxidase as a major source of ROS production in diabetic glomeruli. Similarly, Manea *et al.* [32] have also demonstrated that long-term exposure of pericytes to high glucose results in increased mRNA and protein expression of the p22^{phox} subunit. Thus, the increased production of mRNA for p22^{phox} in our study is an important observation. Moreover, the gene coding for the p22^{phox} is polymorphic [33] and diabetic subjects with the C242T polymorphisms have been reported to be at higher or lower risk of developing diabetic nephropathy [34,35]. Similarly, the T242T genotype of the p22^{phox} C242T polymorphism is associated with decreased risk of severe diabetic retinopathy [36]. Thus, it would be worthwhile to examine the role of these p22^{phox} polymorphisms in the genetic susceptibility to diabetic microvascular complications in Asian Indians.

Additionally, it appears that the metabolic milieu in diabetic patients differentially dictates the formation of the NADPH oxidase complex and its activation. The relationship between the metabolic milieu and induction of the oxidative stress is further substantiated by the finding of positive correlations between p22^{phox}, HbA_{1c}, lipid peroxidation and protein carbonyl levels in the diabetic subjects.

Increased HO-1 expression is an adaptive response in models of oxidative stress-related pulmonary and cardiovascular disease [37,38] and in monocytes from patients with Type 2 diabetes [10]. However, we observed reduced levels of HO-1 mRNA in patients with Type 2 diabetes, particularly in those with microangiopathy. HO-1 levels negatively correlated to p22^{phox}, HbA_{1c}, lipid peroxidation and protein carbonyl levels. Responses of resistance to oxidative stress may be poor in our diabetic patients and this might explain the decreased HO-1 gene expression in our study. In contrast, Avogaro *et al.* [10] have shown increased HO-1 expression in diabetic subjects. Interestingly, the negative association of HO-1 and p22^{phox} expression in our study merits attention because a recent study [39] demonstrated induction of HO-1 inhibiting NADPH oxidase activity in macrophages. Our results are also in agreement with a clinical study [40] in which reduced levels of HO-1 mRNA in skeletal muscle from Type 2 diabetic patients were associated with abnormal insulin-stimulated glucose disposal and with markers of muscle oxidative capacity. Additionally, chronic hyperglycaemia lowers HO-1 levels by interfering with transcription factors in the retina of diabetic rats [41]. Indeed, repression or induction of the expression of HO-1 is a dynamic process and depends on the cellular micro-environment [42]. As HO-1 is inducible by many diverse stimuli including NO, alterations in these stimuli in the diabetic milieu may have a regulatory role in HO-1 induction. These studies and our current observations imply a negative association of HO-1 levels with hyperglycaemia and oxidative damage.

The association of reduced levels of HO-1 with increased blood pressure in our study is of interest because the heme–hemoxygenase system has been implicated in the regulation of vascular reactivity and blood pressure [43]. A polymorphism of the promoter region of the human HO-1 gene is associated with susceptibility to coronary artery disease in Type 2 diabetic patients [44,45].

While the presence of microangiopathy in diabetic subjects was strongly related to reduced HO-1 mRNA and increased protein carbonyl levels, there were no significant differences in p22^{phox} mRNA and TBARS levels in diabetic subjects with and without microangiopathy. There may be plausible reasons for this. Augmentation of NADPH oxidase and lipid peroxidation may represent an early oxidative stress state [46–48] which could be reversible, while increased protein carbonyls represent long-lived cellular changes, connected with duration of diabetes, and hence might appear as markers of diabetic microangiopathy. A definite role for reactive carbonyl compounds in the pathogenesis of diabetic nephropathy has been recently reported [49]. Although oxidative damage markers might have been improved by associated therapy in diabetic patients, we did not detect significant changes in p22^{phox} or HO-1 mRNA expression levels between patients taking only glucose-lowering agents and those taking glucose-lowering agents along with other medication. This apparent lack of change supports the notion that the associated therapy may not directly alter the sources of pro-oxidant or antioxidant molecular signals in diabetic subjects. However, more experimental data on larger numbers of patients are needed to verify this.

Thus, increased p22^{phox} and reduced HO-1 gene expression seen in our study in patients with diabetes and microangiopathy may implicate a proximal defect in the regulatory aspect of oxidative stress. Recent studies indicate that increased oxidative stress in adipose tissue could be an early step in the metabolic

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syndrome [6]. Increased ROS levels and reduced insulin sensitivity were reported in adipocytes exposed *in vitro* to hyperglycaemia [50–52]. Moreover, alterations in ROS production and/or protective mechanisms have been observed in diabetic glomeruli and retina [31,41] and genetic variations in NADPH oxidase were noticed in subjects with diabetic microangiopathy [34,35]. These studies and our observations, taken together, suggest that polymorphisms of genes that are implicated in anti- and pro-oxidative stress, along with functional studies, may be important in assessing the genetic susceptibility to diabetes and its complications.

To conclude, the results from this study provide evidence that genes associated with the production of ROS and antioxidant defence mechanisms are altered in Asian Indian patients with Type 2 diabetes and the changes are more marked in those with microangiopathy. These alterations are associated with markers of oxidative stress, such as lipid peroxidation and protein carbonyls. It is therefore tempting to speculate that therapies that can modulate the increase in cellular ROS may be useful in preventing vascular complications. Suppression of NADPH oxidase was recently shown to be a promising therapeutic strategy because localized adventitial delivery of an NADPH oxidase inhibitor reduced overall vascular O^{2-} and neointima formation [53]. Given the regulatory aspects and cross-talk between HO-1 and NADPH oxidase [39], we suggest that induction of HO-1 by pharmacological means may also be a novel approach to amelioration of oxidative insults in various tissues. Hence, attempts to modulate specifically the sources of ROS production in target tissues by drugs or bioactive molecules may potentially abrogate the deleterious effects of hyperglycaemia and serve as novel therapeutic strategies in diabetic patients.

Competing interests

None declared.

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