Diabetologia (2003) 46:524–530
DOI 10.1007/s00125-003-1061-4

Diabetologia

In situ protein Kinase C activity is increased in cultured fibroblasts from Type 1 diabetic patients with nephropathy

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Abstract

Aims/hypothesis. To verify whether individual susceptibility to diabetic nephropathy resides in an intrinsic difference in Protein Kinase C (PKC) activity.
Methods. We compared the effect of different glucose concentrations on PKC activity, PKC isoform expression and diacylglycerol (DAG) content in cultured fibroblasts from 14 Type 1 diabetic patients who developed nephropathy with those in cells from 14 patients without nephropathy. We recruited 14 normal subjects as control patients. Forearm skin fibroblasts were cultured in either normal (5 mmol/l) or high (20 mmol/l) glucose concentrations.
Results. In normal glucose, in situ PKC activity was higher in Type 1 patients with nephropathy (10.1±1.4 pmol/min/mg protein; $p<0.01$) than in those without (6.8±0.8) and the normal control subjects (6.3±0.5). This difference was due to increased concentrations of PKCα isoform in the membrane fraction of fibroblasts from patients with nephropathy. DAG content was also higher in cells from Type 1 patients with nephropathy. Incubation in high glucose concentration caused a further increase in PKC activity and DAG content in quiescent fibroblasts from patients with diabetic nephropathy, with no significant changes in cells from diabetic patients without nephropathy and normal control subjects.
Conclusion/interpretation. Differences in PKC activation could contribute to the individual susceptibility to renal damage in Type 1 diabetic patients. [Diabetologia (2003) 46:524–530]

Keywords Diabetic nephropathy, hyperglycaemia, human fibroblasts, protein kinase C, diacylglycerol, type 1 diabetes.

Kidney disease is one of the most serious and costly complications of diabetes. However, the kidney does not fail in all people with diabetes. The incidence of diabetic nephropathy increases up to 17 years from diagnosis of Type 1 diabetes and then sharply declines [1]. Hyperglycaemia, though critically necessary for kidney disease development, is insufficient to totally account for it. Predisposition to hypertension and cardiovascular disease could be an important determinant of susceptibility to renal disease [2, 3]. The familiar clustering of diabetic nephropathy both in insulin-dependent [4] and non-insulin-dependent diabetic patients [5] is consistent with the possibility that genetic factors might explain the liability to or protection from renal disease of diabetic patients. Overt diabetic nephropathy is the result of complex multifactorial processes that are affected by an interaction of environmental, familial and genetic influences. To get closer to the possible genetic basis, a possible approach is the search of intermediate phenotypes, associated with diabetic renal disease.

We and others have shown that skin fibroblasts from insulin-dependent diabetic patients with neph-

Received: 3 September 2002 / Revised: 19 November 2002
Published online: 26 March 2003

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Abbreviations: PKC, Protein Kinase C; DAG, diacylglycerol; AER, albumin excretion rate; TPA, tetradecanoyl-13-phorbol acetate; TCA, trichloride acid.

ropathy have enhanced activity of the sodium-hydrogen antiport and increased ^{3}H-thymidine incorporation into DNA compared to fibroblasts obtained from diabetic patients without nephropathy and normal control subjects [6, 7, 8]. These results, also confirmed in immortalized lymphoblasts [9], suggested that abnormalities in cell function could be independent of the metabolic disturbances of diabetes and intrinsic to insulin-dependent diabetic patients who develop kidney disease.

Protein kinase C (PKC) is a family of serine-threonine protein kinase isoforms that can be subdivided into three different subfamilies on the basis of their structural homology and cofactor requirement. PKC activation regulates a variety of cellular functions including permeability, contractility, cellular proliferation, basement membrane biosynthesis and responsiveness to cytokines and hormones [10, 11]. There is increasing evidence that increased extracellular glucose concentrations can activate several isoforms of protein kinase C as a consequence of a glucose-driven increase in de novo synthesis of diacylglycerol [10, 11]. Cultured skin fibroblasts have proven to be a useful tool for investigation of various genetic errors of metabolism and our earlier results suggest that these cells could help to identify intrinsic abnormalities of cell function which might participate in the pathogenesis of diabetic cardiorenal complications [6, 7, 8, 12].

We report the activity and isoform expression of PKC in quiescent skin fibroblasts, cultured in normal- and high-glucose concentrations, from Type 1 (insulin-dependent) diabetic patients with or without nephropathy and from normal non-diabetic control subjects.

Materials and Methods

Patients. We recruited 14 Type 1 (insulin-dependent) diabetic patients with overt diabetic nephropathy, defined as an urinary albumin excretion (AER) persistently greater than 200 μg/min in sterile urine, duration of diabetes more than 10 years, and concomitant retinopathy, from the outpatient clinic at Padua's Hospital. We also recruited 14 normal subjects and 14 long-term Type 1 diabetic patients without a family history of hypertension and of diabetes with normal albumin excretion rate (AER <20 μg/min) who served as control subjects. The two diabetic groups came from the same clinical cohort and had similar age, diabetes duration, BMI and sex distribution. Non-diabetic controls were younger but otherwise comparable to the diabetic patients (Table 1). All subjects were of Caucasian origin and gave their informed consent to take part in the study, which was approved by the Committee on Ethical Practice of Padua's Hospital.

On the morning of the skin biopsy height and weight were recorded without shoes and in light indoor clothing, and blood was taken for determination of glycosylated haemoglobin (Corning gel electrophoresis, Ciba-Corning, Calif, USA) and serum creatinine (Jaffe reaction rate method, Hitachi autoanalyser, Hitachi, Japan). Arterial blood pressure was measured with a standard mercury sphygmomanometer, to the nearest 2 mmHg, in the dominant arm after at least 10 min rest in the supine position. Mean blood pressure was calculated as diastolic blood pressure plus one third pulse pressure. GFR was measured by plasma clearance of ^{51}Cr-EDTA within a month of the skin biopsy in the diabetic patients only [13]. We collected three timed overnight urine samples for measurement of urinary AER and the median value was used for classification.

We calculated the mean of all HbA_{1c} values obtained over 6 years before the skin biopsy as a measure of long term metabolic control for each patient. A median of ten HbA_{1c} measurements per patient were retrieved from the medical records with a range from 6 to 24.

All patients with proteinuria were on anti-hypertensive medication, which included calcium antagonists, angiotensin converting enzyme inhibitors, vasodilators and loop diuretics either alone or in combination. The patients with normoalbuminuria were taking no drug other than insulin. The diabetic patients withheld their morning insulin injection until after the skin biopsy and the patients taking antihypertensive drugs were asked to stop them at least 36 h before the study.

Skin fibroblast cultures

Skin biopsies were taken by excision under local anaesthesia from the anterior surface of the forearm. Fibroblasts were cultured in HAM'S F-10 Nutrient Mixture (Sigma Aldrich Chem-

Table 1. Clinical features of Type 1 (insulin-dependent) diabetic patients with and without nephropathy and normal control subjects. Values are means ± SEM, except for albumin excretion rate which is given as median and range

	Type 1 diabetic patients with nephropathy	Type 1 diabetic patients without nephropathy	Normal control subjects
No. (male/female)	10/4	9/5	11/3
Age (yr)	41±5	42±6	35±4
BMI (Kg/m^2)	23±3	23±2	24±3
Duration of diabetes (yr)	25±7	24±6	–
Insulin dose (U/day)	43±5	40±5	–
Glycosylated haemoglobin (%)	8.8±0.4	9.0±0.6	–
Mean blood pressure (mmHg)	97±6[a]	88±4	86±4
Albumin excretion rate (μg/min)	860 (302–4047)	7 (1–16)	6 (4–15)
Serum creatinine (μmol/l)	110±10[a]	90±6	87±4
Glomerular filtration rate(ml/min/1.73 m^2)	92±7 [a]	121±5	–

[a] $p<0.01$ vs normal controls and Type 1 diabetic patients without nephropathy

ical, Irvine, UK), supplemented with 10% foetal bovine serum (Hyclone NL), 4 mmol/l glutamine (Sigma Aldrich), 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma Aldrich) at 37°C until confluence. After the fourth passage, cells were harvested and stored in liquid nitrogen. For each experiment fibroblasts were thawed and grown as described previously in either normal (5 mmol/l) or high (20 mmol/l) glucose for 72 h. All studies were carried out at the 5 to 7th passage.

PKC activity in situ

Fibroblasts were seeded into 96-well plates at a density of 20 000 cells per well. Cells were cultured in either normal (5 mmol/l) or high (20 mmol/l) glucose for 72 h. The growth medium was changed with quiescent medium (serum-free) 24 h before the experiment. The medium was aspirated and fibroblasts were washed with PBS (Phosphate buffer saline pH 7.4). Cells were incubated with quiescent medium or 1 µmol/l TPA for 20 minutes [14]. Each experiment was conducted in quadruplicate. The test media were aspirated and replaced with 40 µl of the buffered salt solution containing 137 mmol/l NaCl, 54 mmol/l KCl, 10 mmol/l magnesium chloride, 0.3 mmol/l sodium phosphate, 0.4 mmol/l potassium phosphate, 25 mmol/l β-glicerophosphate, 5.5 mmol/l D-glucose, 5 mmol/l EGTA, 1 mmol/l calcium chloride, 20 mmol/l HEPES (pH 7.2 30°C), 100 µmol/l digitonin and 50 µmol/l [γ-^{33}P] ATP (3000 cpm/pmol) (ICN Biomedical Costa Mesa, Calif., USA). A 100 µmol/l PKC-specific peptide substrate (VRKRTLRRL-Sigma Aldrich) was added to the buffer.

The kinase reaction proceeded for 10 min at 30°C before termination by the addition of 10 µl of 25% (w/v) trichloride acid (TCA). 45 µl of the reaction mixture were spotted onto 2 cm phosphocellulose circles (Wathman p-81, Wathman, Clifton, N.J., USA) and washed three times with 75 mmol/l phosphoric acid and once with 75 mmol/l sodium phosphate pH 7.5 [14, 15].

Due to the basicity of VRKRTLRRL substrate, it is retained by the phosphocellulose filter at neutral pH, whereas contaminating free [γ-^{33}P] ATP is removed, reducing the assay blank.

The PKC-dependent phosphorylation of the peptide substrate bound to the filter was quantified by scintillation counting with a Packard β-counter. Fibroblast proteins per well were solubilized in 0.1% SDS, 0.1 N NaOH and quantified by using the method of Lowry.

The background phosphorylation was assessed in two ways. Firstly, to assess background phosphorylation of the VRKRTLRRL peptide substrate, immediately before adding the reaction buffer, 40 µl of 10% ice-cold TCA was added to the cell monolayer for 10 min to precipitate cell proteins and eliminate the possibility of kinase activity. TCA was then aspirated, the reaction buffer added to measure kinase independent phosphorylation of the VRKRTLRRL peptide. Secondly, to assess whether there was a kinase dependent background phosphorylation of cell proteins other than the VRKRTLRRL, fibroblasts were incubated without the specific substrate.

Immunoblot analysis of PKC isoforms

Cytosol and membrane fractions from cultured fibroblasts were immunoblotted with rabbit polyclonal antibodies for PKC α, β_2, δ, ε and ξ (Santa Cruz Biotechnology, Santa Cruz, Calif., USA). Cytosol and membrane protein extracts (30 µg) were solubilized in Laemmli buffer and then separated by electrophoresis through a 10% polyacrylamide gel. Proteins separated on the gels were electroblotted onto nitrocellulose membranes (Hybond ECL; Amersham Pharmacia Biotech, Buckinghamshire, UK) in Tris-glycine transfer buffer with 20% methanol for 2 h at 100 V in the cold, using a BioRad Transblot cell. The membranes were blocked overnight at 4°C in PBS containing 0.05% (vol/vol) Tween (T-PBS) and 5% BSA. Membranes were exposed to primary antibody (1:2,000 dilution) for anti-PKC isoforms overnight at 4°C. Membranes were washed (4× for 20 min) with the same buffer and then incubated with 1:4,000 goat anti-rabbit antibody conjugate to horseradish peroxidase. Detection was made using the enhanced chemiluminescence (ECL) system from Amersham. Blots were scanned and quantified with a BioRad Chemiluminescence Molecular Imaging System, and results were expressed relative to the control, on the same blot, set at 100%.

Assay of total DAG contents. Fibroblasts were seeded onto six-well plates at a density of 10^5 cells per well.

Cells were incubated for 72 h with normal (5 mmol/l) and high (20 mmol/l) glucose concentration. Then 24 h before diacylglycerol (DAG) extraction, the culture medium was replaced with quiescent medium (serum free). Cells were washed three times with PBS (phosphate buffer saline pH 7.4) and harvested in 0.5 ml cold 1 mol/l NaCl. Samples were extracted by a modification of a known method [16]. DAG was extracted with 1.5 ml chloroform:methanol 1:2 (v/v). The monophase was mixed and 0.5 ml NaCl plus 0.5 ml chloroform were added.

After centrifugation at 5,000 g for 2 min, the lower chloroform phase was evaporated under nitrogen. The samples were stored at −20°C until the DAG assay.

The DAG concentration was measured using a commercially available DAG assay system specific for sn-1,2 diacylglycerol (Amersham). The method is based on the DAG kinase mediated formation of [^{33}P] phosphatidic acid from DAG and [^{33}P]-γ-ATP (ICN Biomedical Costa Mesa, Calif., USA) [17, 18]. The DAG content of each sample was calculated using a standard curve and normalized to cell number.

Statistical analysis. Statistical calculation was carried out using a Statistical Package for the Social Sciences (SPSS-PC, Chicago, Ill., USA). Differences between group means were tested by analysis of variance, with post-hoc test of Dunnet for comparisons between groups. The relationship between variables was tested with linear regression analysis. Skewed data (albumin excretion rate) were logarithmically transformed. A two-tailed *p* value of less than 0.05 was considered statistically significant. Data are shown as means ± SEM unless otherwise stated.

Results

Diabetic patients with and without nephropathy had similar ages, sex distribution, duration of diabetes and BMI and required similar doses of insulin. Non-diabetic control subjects were slightly younger, but BMI and sex distribution were similar to the diabetic patients. HbA_{1c} levels over 6 years were similar in patients with nephropathy and in those without (Fig. 1). Blood pressure was higher in those with nephropathy when compared to those without nephropathy and normal control subjects. There was a family history of hypertension in seven out of the fourteen patients with nephropathy. All Type 1 diabetic patients

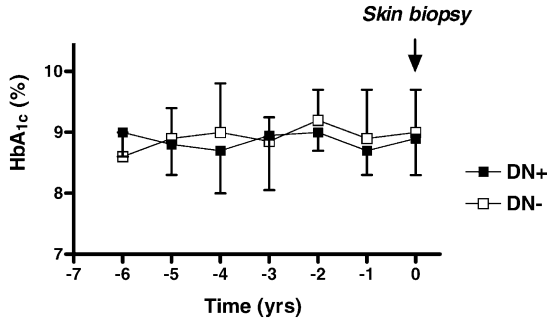

Fig. 1. Glycosylated haemoglobin (HbA_{1c}) (means ± SEM) in Type 1 diabetic patients with (DN +) and without nephropathy(DN –) over the last 6 years before skin biopsy

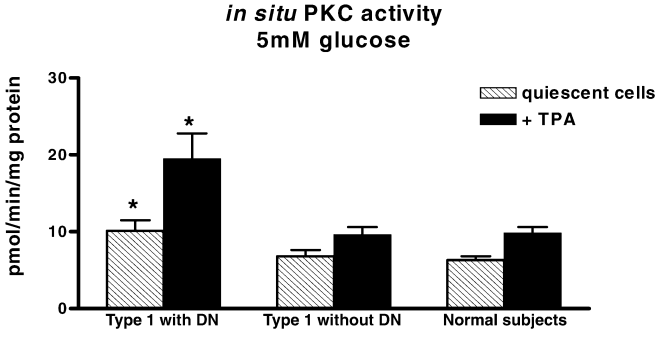

Fig. 2. In situ PKC activity in cultured skin fibroblasts from Type 1 diabetic patients with and without nephropathy and normal control subjects. PKC activity was measured in quiescent cells (dashed bars) and in TPA stimulated cells (solid bar). *p<0.01 vs the corresponding values measured in Type 1 diabetic patients without nephropathy and normal control subjects

with nephropathy were hypertensive (blood pressure ≥140/90 mmHg) before the development of overt proteinuria. In the group with nephropathy serum creatinine was higher and GFR was lower (Table 1), but all patients still had GFR values above 60 ml/min/1.73 m^2.

In situ PKC activity in normal (5 mmol/l) and high glucose medium (20 mmol/l). The specific in situ PKC activity in normal glucose medium was higher in quiescent fibroblasts from Type 1 diabetic patients with nephropathy (10.1±1.4 pmol/min/mg protein; p<0.01) compared with that of Type 1 diabetic patients without nephropathy (6.8±0.8) and normal control subjects (6.3±0.5) (Fig. 2). Exposing fibroblasts to TPA activated PKC in all subjects, but, again, the response to TPA was greater in Type 1 diabetic patients with nephropathy compared with that of Type 1 diabetic patients without nephropathy and normal control subjects (Fig. 2).

In order to evaluate the time-course of glucose-induced PKC activation, PKC activity was measured as a function of time in three subjects in each group. At all time points, PKC activity remained constant in fibroblasts from diabetic patients without nephropathy

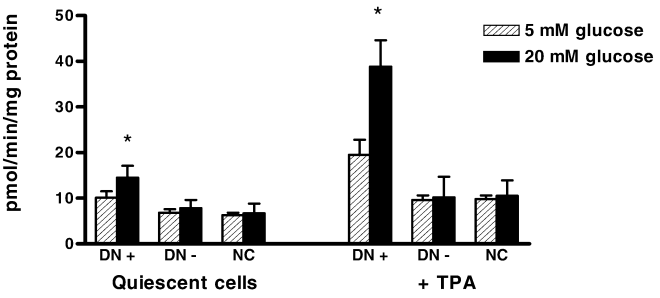

Fig. 3. Effect of different glucose concentrations (5 mmol/l, dashed bars and 20 mmol/l solid bars) on in situ PKC activity in cultured skin fibroblasts from Type 1 diabetic patients with (DN +) and without nephropathy (DN –) and normal control subjects (NC). PKC activity was measured in quiescent cells and in TPA stimulated cells

and in normal control subjects. On the contrary, in high glucose medium, PKC activity was increased in fibroblasts from diabetic patients with nephropathy after 48-h incubation compared to that observed in normal glucose medium (data not shown). The maximal activation was found after 72-h incubation in high glucose. The following experiments in all subjects were therefore carried out after 72-h incubation in high glucose medium.

Figure 3 shows that increasing glucose concentrations from 5 to 20 mmol/l increased the specific PKC activity (from 10.1±1.4 pmol/min/mg protein to 19.5±3.3; p<0.001)only in quiescent fibroblasts from diabetic patients with nephropathy. No significant changes were observed in the cells from the other two groups. Similarly, in high glucose media, TPA-activation of PKC activity was greater than that achieved in normal glucose only in cells from Type 1 diabetic patients with nephropathy (Fig. 3).

The increase in PKC activity in diabetic patients with nephropathy was not caused by differences in cell density as assessed by cell counts which was similar in the three groups (data not shown). In the whole cohort of diabetic patients, PKC activity in fibroblasts was not related to GFR, serum creatinine or HbA_{1c}. In the patients with nephropathy no relationships were found between PKC activity and GFR, albumin excretion rate, HbA_{1c} or blood pressure values.

Immunoblot analysis of PKC isoenzymes in normal (5 mmol/l) and high glucose medium (20 mmol/l). Immunoblot analysis carried out on cytosol and membrane fractions of cultured fibroblasts in quiescent state showed only one calcium-dependent PKC isoform, PKCα. We did not detect any specific immunoreactivity for PKCβII on cytosol or membrane fractions.

In normal glucose media, no differences were observed in the expression of the PKC isoform ε and ξ in fibroblasts from Type 1 diabetic patients with and without nephropathy and the normal controls in the

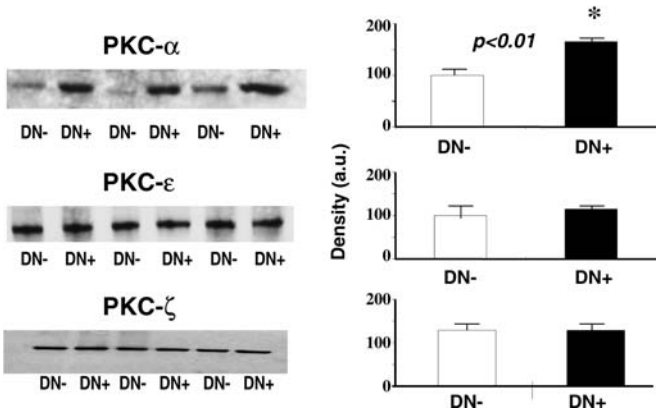

Fig. 4. Left panel: representative immunoblots of PKC isoform expression in the membrane fraction of cultured skin fibroblasts from three Type 1 diabetic patients with (DN +) and three without nephropathy (DN −). Right panel: densitometry measurements expressed as percent of normal control subjects (taken as 100%) for each PKC isoform shown on the left panel. *p <0.05 vs patients without nephropathy

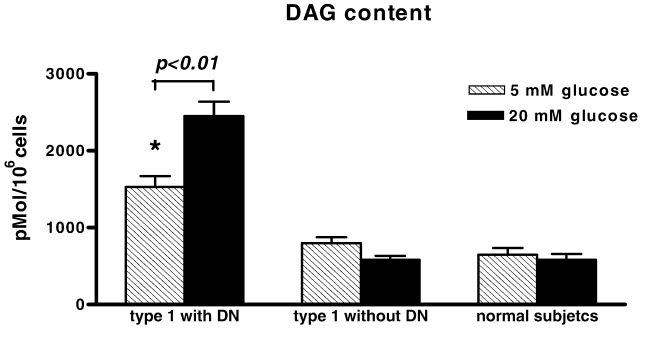

Fig. 5. Effect of different glucose concentrations (5 mmol/l, dashed bars and 20 mmol/l solid bars) on total DAG content in cultured skin fibroblasts from Type 1 diabetic patients with and without nephropathy and normal control subjects

membrane (Fig. 4) and in the cytosol fraction. In contrast, there was an increase in the expression of PKCα isoform in the membrane fraction of fibroblasts from Type 1 diabetic patients with nephropathy in comparison with that found in the patients without nephropathy and in normal controls (Fig. 4). PKCα content in the cytosol fraction was similar in patients with nephropathy and in those without.

In high glucose media, the expressions of PKCα isoforms in the membrane fraction of fibroblasts from Type 1 diabetic patients with nephropathy was higher than in the other two groups, but without any increase when compared with those found in normal glucose medium. Similarly no difference in the cytosol fraction was observed in the three groups of subjects.

Total DAG content – in normal (5 mmol/l) and high glucose medium (20 mmol/l). Total DAG content in normal glucose medium was increased in cultured fibroblasts from Type 1 diabetic patients with nephropathy (1528±143 pmol/10^6 cell; p<0.01) in comparison with those measured in cells from diabetic patients without nephropathy (798±78) and normal control subjects (648±87). An increasing glucose concentration from 5 to 20 mmol/l increased total DAG content only in cultured fibroblasts from diabetic patients with nephropathy, while they were comparable to those measured in normal glucose medium in cells from the other two groups (Fig. 5).

Discussion

This study shows an enhanced in situ PKC activity in quiescent skin fibroblasts of Type 1 patients with nephropathy cultured in normal glucose concentrations. Moreover, high glucose concentrations increased PKC activity in cells from diabetic patients with nephropathy, exaggerating the difference already present in a normal glucose concentration between cells from Type 1 diabetic patients with nephropathy and those from diabetic patients without nephropathy and non-diabetic control subjects. Using antibodies specific to the various protein kinase C isoforms, the measured increase in protein kinase C activity observed in fibroblasts from diabetic patients with nephropathy was mostly due to increased PKCα expression in the membrane fraction, whereas the other isoform expression was unchanged. This increase in membrane PKCα suggests an enhanced translocation of this isoform the cytosol to the membrane. This increased translocation with a greater total PKC activity was associated with an increased total DAG content in fibroblasts from Type 1 diabetic patients with nephropathy. Altogether, these results suggest that the abnormal PKC regulation in fibroblasts cultured for several passages in vitro from Type 1 diabetic patients with nephropathy is a distinct phenotype feature that could be genetically determined.

In vivo hyperglycaemia in diabetic patients is unlikely to explain the results of our study: HbA_{1c} levels over the last 6 years before the skin biopsy was similar in Type 1 diabetic patients with nephropathy and those without, although a previous poorer glycaemic control cannot be excluded in patients with nephropathy. Furthermore, PKC activity and expression and DAG content were virtually identical in patients without nephropathy and in normal control subjects. We have recently shown that PCK activity in human monocytes is acutely up-regulated by plasma glucose concentrations in vivo both in normal and Type 2 diabetic patients [19]. In that study, however, not only actual glucose concentrations, but also insulin sensitivity seemed to affect PKC activity, thus suggesting that alterations in PKC activity could take place over a long time.

This might be relevant in our study since Type 1 diabetic patients with nephropathy are also more insu-

lin resistant than those without [20]. The PKC has been directly implicated in insulin signalling and increased PKC activity in liver, muscle and adipose tissue have been associated with insulin resistance [10]. Transfected hepatocytes that over-express PKC become insulin resistant [21]. Thus a role of insulin resistance in determining long-term phenotypic changes in cells derived from our Type 1 diabetic patients with nephropathy cannot be excluded.

Although PKC activity and DAG content were already higher in fibroblasts from Type 1 diabetic patients with nephropathy in normal glucose concentrations, high glucose concentrations further increased PKC activity and DAG content, thereby providing a biochemical explanation of the interaction between diabetic milieu and intrinsic features of individual patients. It is of note that high glucose concentrations increased DAG and PKC only after 72 h of exposure in keeping with the results found in other cell models [22]. It was suggested that high glucose has other effects on PKC besides stimulating the translocation of PKC by enhancing DAG synthesis.

The cause for the increased cellular DAG content was not investigated in this study. It is possible that an increased glucose uptake could be responsible for the abnormal DAG content and PKC activity in fibroblasts from Type 1 diabetic patients with nephropathy [23]. An increased glucose transport and GLUT1 cell-surface content was found in fibroblasts from Type 2 diabetic patients [24]. The authors suggested that the abnormalities in fibroblasts glucose uptake could represent a marker for insulin resistance in Type 2 diabetes. Evidence is also available that, in vivo, non-insulin-dependent glucose uptake is abnormally increased in forearm muscle of diabetic subjects as compared to non-diabetic individuals at the same glucose concentrations [25]. Again all these data might be relevant to our study, in that insulin resistance was found to be consistently associated with abnormal albumin excretion both in Type 1 [20] and Type 2 diabetic subjects [26].

Although amongst the many isoforms of PKC, PKCβII isoform has been reported to be predominantly activated by hyperglycaemia in all vascular tissues [11], we did not detect any specific immunoreactivity for PKCβII in fibroblasts from normal and diabetic subjects, as reported by others [27]. It is known that the specific PKC isoform activated by hyperglycaemia varies across tissues and species [28].Thus, involvement of PKC in the vascular dysfunction of diabetes might depend on the bed studied. In the renal glomeruli of diabetic rats augmentation of α, β, δ and ε PKC activity has been noted [29]. Furthermore it has been reported that high glucose concentrations lead to an increased expression of PKCα, but not of PKCβII in endothelial cells [30] . In these cells, the increased permeability induced by high glucose was selectively blocked by specific antisense oligonucleotides [31]. We found that high glucose concentration increased PKC activity without any changes in PKC isoforms expression. An explanation for this observed dissociation between protein content and activity after incubation in high glucose is not readily apparent. It could indicate that the enhanced PKC activity cannot be attributed to an increased membrane density of PKC molecules, but to an increased turnover number as already observed for other membrane proteins [9]. The possibility, however, that other PKC isoforms might have been translocated by hyperglycaemia cannot be totally excluded.

The effects of antihypertensive therapy in the diabetic patients with nephropathy, even if it persisted after several cell passages in vitro, is also unlikely to explain the differences among the groups. Moreover, PKC activity was similar in the patients taking only one antihypertensive drug as compared to those taking two or more drugs. The available evidence from animal studies would suggest that some of the antihypertensive drugs used, particularly the ACE inhibitors, would reduce PKC activity [32]. Thus, if anything, antihypertensive therapy would tend to reduce rather than enhance the difference. It is also unlikely that the increased PKC activity in diabetic patients with nephropathy is secondary to the impairment in renal function since no relationship was observed between the decline in GFR and PKC activity.

All patients with nephropathy were hypertensive and 50% had a positive family history for hypertension. That a predisposition to essential hypertension confers an increased susceptibility to renal damage in diabetic patients is well established [2, 3]. An altered PKC activity was described both in hypertensive patients and in animal models of essential hypertension [33, 34]. This supports the possibility that PKC might play a role in the pathogenesis of hypertension in diabetic patients at risk of renal disease.

All these observations of stable phenotypic changes in cell function, despite serial passaging of cells in identical media in vitro, suggest a likely intrinsic component in the cell-altered response to diabetes in the subset of patients at risk of kidney disease.

Although further studies are required to identify the primary cellular mechanism responsible for altered PKC activity in cells from patients with nephropathy, it is remarkable that cultured skin fibroblasts represent a useful in vitro model not only for the identification of patients at risk of developing diabetic renal disease, but also for a better understanding of the complex multifactorial mechanism leading to long term organ damage in diabetes.

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