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Glycolytic enzyme expression and pyruvate kinase activity in cultured fibroblasts from type 1 diabetic patients with and without nephropathy

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ABSTRACT

Since type 1 diabetes mellitus (T1DM) patients with nephropathy (DN+) are insulin-resistant, we aimed to identify (new) potential molecular sites involved in the alterations of glucose metabolism in these patients. We examined the expression of glycolytic enzymes in cultured fibroblasts from T1DM(DN+) patients as compared to those from T1DM patients without nephropathy (DN−) and from controls. Pyruvate kinase (PK) activity was also determined. Human skin fibroblasts were grown in normal glucose (6 mM). RNAs and proteins were analyzed, respectively, using cRNA microarray and two-dimensional electrophoresis followed by identification with mass spectrometry. PK activity was measured using a spectrophotometric assay. As compared to controls, increases in the gene expression of hexokinase, phosphoglucomutase, phosphofructokinase, aldolase and triosephosphate isomerase were found in T1DM(DN+) patients, but not in T1DM (DN−) patients. In T1DM(DN+) patients, the protein analysis showed an altered expression of three glycolytic enzymes: triosophosphate isomerase, enolase and PK. In addition, PK activity in fibroblasts from T1DM(DN+) patients was lower than that in T1DM(DN−) and in controls. In conclusion, this study reports novel alterations of enzymes involved in glucose metabolism that may be associated with the pathophysiology of insulin resistance and of renal damage in T1DM(DN+) patients.

1. Introduction

Diabetic nephropathy (DN) is a chronic complication of T1DM, affecting about 30% of all diabetics over their lifetime [1]. DN carries a heavy burden for both the affected patients and the health system, and it is a potentially life-threatening condition. Therefore, both the early detection of DN and of risk factors are necessary for preventive and therapeutic strategies.

The onset and development of DN are thought to be genetically-determined [1]. The detection of phenotypic markers associated with a predisposition to disease could be accomplished by comparing patients with long standing diabetes (i.e. >10 years from diagnosis) who have developed DN with diabetic patients who did not develop DN after a similar disease duration, despite a comparable exposure to environmental risk factors.

T1DM(DN+) patients are typically more insulin-resistant than either T1DM(DN−) patients or non-diabetic control subjects [2]. Although the mechanism(s) leading to insulin resistance in these subjects is not completely understood, it is generally believed that it is related to proteinuria and/or the impairment of renal function [3]. Insulin resistance reveals disturbances of intracellular glucose metabolism which can shift the effect of insulin from physiological to pathological signalling pathways [4,5]. Therefore, knowledge of intracellular steps of glucose metabolism, that are altered in patients with chronic complications, can shed a new light on the pathogenesis of diabetic complications and/or on associated metabolic defects.

Detection of differentially-expressed enzymatic proteins in cells, tissues, or biological fluids, (derived from patients with and without established DN and from healthy controls) can highlight specific markers and/or biochemical steps associated with DN.

Ideally, the cells to be studied would be those isolated from renal tissues, such as glomerular cells, mesangial or tubular cells, and podocytes; however, these cells are difficult to collect from living human subjects. Instead, cultured skin fibroblasts have proven to be a useful alternative tool for the investigation of a number of putative pathophysiological mechanisms of disease [6]. Moreover, fibroblasts are involved in the pathogenesis of renal sclerosis [7], and are responsive to insulin [8]. Phenotypes found to be associated with DN in cultured human fibroblasts include increased DNA synthesis, increased activity of Na^+/H^+ antiport, greater PKC activity, and lower

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Ca-pump-mediated Ca^{2+} efflux [5,9]. Each of these factors could potentiate cell growth. Also, these differences persist after several cell passages in cultured media with normal glucose concentrations, suggesting that they are intrinsically connected to a constitutive genetic predisposition and not to the diabetic milieu itself.

The profiling of the whole transcriptome of human fibroblasts from normal controls, as well as T1DM(DN+) and (DN−) patients, can allow for the identification of gene(s) related to DN. In addition, it is desirable to complement the mRNA data with data on protein expression, since transcriptional and translational data do not always correlate [10,11].

Such a comparative study has yet to be conducted in cells from T1DM patients with and without established nephropathy.

We have therefore compared the proteomic and transcriptomic profiles of glycolytic enzymes from long-term T1DM(DN+) and (DN−) subjects, aiming to detect new markers of the impaired glucose disposal usually found in T1DM(DN+) patients [2].

2. Materials and methods

2.1. Chemicals and reagents

Methanol, acetic acid, TRIS, glycerol and glycine were from Carlo Erba (Rodano, MI, Italy). Dithiothreitol (DTT), Pharmalyte pH 3–10 and 4–7 and Immobilised pH gradient (IPG) strips, were purchased from Amersham Biosciences (Uppsala, Sweden). Acrylamide and ammonium persulfate were from Eurobio (Courtaboeuf Cedex, France). All other chemicals and enzymes were from Sigma (St. Louis, MO, USA).

2.2. Subjects

Five long-term T1DM(DN+) patients (2 men and 3 women; age: 35.6±3.8 years) with a urinary albumin excretion rate (AER) of >200 μg/min, five T1DM(DN−) patients (2 men and 3 women; age: 39.4±5 years) (AER: <20 μg/min) and five healthy control volunteers (2 men and 3 women; age: 37±4.6 years) without a family history of hypertension or diabetes, were recruited. All subjects were of Caucasian origin with comparable BMI (22.9±1.2 in controls, 24.1±0.4 in T1DM(DN−), 22.2±1.2 in T1DM(DN+)) mean blood pressure (93.3±10 in controls, 95.2±2.7 in T1DM(DN−), 99.1±6.8 in T1DM(DN+)). Disease duration (22.8±3.2 in T1DM(DN−), 21.8±4.5 in T1DM(DN+)) and HbA_{1c} (9.3±0.8 in T1DM(DN−); 10.5±1.3 in T1DM (DN+)) were comparable between the two groups of diabetic patients.

Two of the five microalbuminuric patients had some degree of renal insufficiency (creatinine concentrations of 588 and 860 μmol/L), however none of the nephropathic patients were in a condition of end stage renal disease.

All drugs were suspended the day before the study. The aims of the study were explained in detail, and each subject signed an informed consent. The protocol was approved by the Ethics Committee of the Medical Faculty at the University of Padova, Italy, and was performed according to the Helsinki Declaration (1983 revision).

2.3. Skin fibroblast cultures

Skin biopsies were taken by excision under local anaesthesia from the anterior surface of the forearm, and the fibroblasts were cultured in normal glucose (6 mmol/L) as described previously [5]. The growth medium was changed with quiescent medium (serum-free) 24 h before the protein extraction. Cells were used between the 7th and the 8th passage.

2.4. Sample preparation

The quiescent medium was aspirated and fibroblasts were washed with PBS. Subsequently, cells were lysed in a buffer containing 8 M urea, 4% 3-3-cholamidopropyldimethylammonio-1-propanesulflonate (CHAPS), 2% IPG buffer pH 3–10 (or pH 4–7) and a cocktail of protease inhibitors. The samples were freeze–thawed, sonicated, concentrated, and desalted by ultrafiltration (Centricon YM-3, Millipore Corporation, Bedford, USA). Protein concentration was determined by Bradford assay [12].

2.5. Two-color microarray-based gene expression

Total RNA was isolated from fibroblasts using a combination of Trizol (Invitrogen Discovery Sciences, Madison, USA) and RNeasy Mini (Qiagen, Milan, Italy) kits. For the microarray assay the RNA quality must be optimal, and the integrity of RNA was systematically checked with use of the lab-on-chip technology in an Agilent Bioanalyzer 2100 with the RNA6000 Nano Assay (Agilent Technologies, Palo Alto, CA). Furthermore the purity was determined by spectrophotometric readings at 260/280/230 nm. cRNA was synthesized from 500 ng of total RNA using the Low RNA Input Linear Amplification Kit and the Two-Color RNA Spike-In (Agilent Technologies), according to the manufacturer's instructions, as described previously [13]. RNA from five healthy control volunteers was collected and used to generate a pool of control RNA. The control and the sample pools were labelled using Cyanine 3 (cy3) and Cyanine 5 (cy5) respectively. In order to control for gene specific dye biases and for dye intensity differences, replicates were included in the experimental design.

The Labelled/Amplified cRNA was purified using the Qiagen's RNeasy mini spin columns. The purified cRNA was read at the spectrophotometer to measure cy3 RNA and cy5 RNA concentrations (pmol/μL), absorbance ratios (260 nm/280 nm), and cRNA concentration (ng/μL).

The samples were hybridized on an oligomicroarray chip (Whole Human Genome Microarray Kit, 4×44K, G4112F Agilent Technologies), which contains about 44,000 60-mer in situ synthesized sequences that comprise the whole human genome. Hybridization was performed only if the yield was >750 ng and the specific activity was >8.0 pmol cy3 or cy5 per μg cRNA, using the Gene Expression Hybridization Kit (Agilent Technologies). The chip was incubated in a rotor oven at 65 °C for 17 h.

2.6. Chip scanning and data analysis

The chip was scanned using a dual-laser Microarray Scanner System (Agilent Technologies). After generating the microarray scan images, images were extracted using the Feature Extraction 9.1 software (Agilent Technologies) and data from different microarray experiments were compared using Rosetta Resolver (Rosetta Biosoftware, Seattle, WA) [13].

2.7. Two-dimensional electrophoresis (2-DE)

2-DE was performed as previously described [14]. Briefly, the extracted proteins were diluted in a rehydration buffer consisting of 8 M urea, 2% CHAPS, 0.5% IPG buffer, 1% DTT to a final volume of 450 μL. Isoelectric focusing was carried out on 24 cm IPG strips (pH range 3–10 and 4–7) using the Ettan™ IPGphor Isoelectric focusing Unit (Amersham) for 38 kVh. Second-dimension SDS-PAGE was performed in 12% acrylamide gels (26×20 cm) using the Ettan DALT six Large Vertical Electrophoresis System (Amersham). The protein samples obtained from each subject (both the patients' and the control group) were run in duplicate (technical replicates).

The 2-DE gels were stained with 0.1% Coomassie Brilliant Blue G250, and scanned on a scanner with 16 bit dynamic range and 300 dpi resolution (Epson Expression 1680 Pro, Seiko Corporation, Japan). Image analysis was done using the Proteomweaver® software (Bio-Rad, Hercules, CA, USA).

Table 1
Gene expression analysis

Gene name	Gene description	Accession no.	IDDM(DN−) vs *N*		IDDM(DN+) vs *N*	
			Fold change	*p* value	Fold change	*p* value
HK1	Hexokinase 1, nuclear gene encoding mitochondrial protein, transcript variant 5	NM_033500	1.44	0.000	1.00	1.000
HK1	Hexokinase 1, nuclear gene encoding mitochondrial protein, transcript variant 3	NM_033497	1.00	1.000	1.51	0.000
HK2	Hexokinase 2	NM_000189	1.06	0.556	**2.04**	0.002
HK3	Hexokinase 3 (white cell), nuclear gene encoding mitochondrial protein	NM_002115	1.00	1.000	1.00	1.000
PGM1	Phosphoglucomutase 1	NM_002633	1.42	0.000	**2.08**	0.000
PGM3	Phosphoglucomutase 3	NM_015599	1.15	0.066	1.45	0.005
GPI	Glucose phosphate isomerase	NM_000175	−1.27	0.025	1.37	0.000
PFKP	Phosphofructokinase, platelet	NM_002627	1.07	0.365	1.75	0.000
PFKM	Phosphofructokinase, muscle	NM_000289	−1.20	0.013	−1.20	0.025
PFKL	Phosphofructokinase, liver, transcript variant 1	NM_001002021	−1.28	0.014	**2.10**	0.000
FBP2	Fructose-1,6-bisphosphatase 2	NM_003837	1.00	1.000	1.00	1.000
FBP1	Fructose-1,6-bisphosphatase 1	NM_000507	1.00	1.000	1.00	1.000
ALDOA	Aldolase A, fructose-bisphosphate, transcript variant 1	NM_000034	1.06	0.438	**2.61**	0.000
ALDOA	Aldolase A, fructose-bisphosphate, transcript variant 2	NM_184041	−1.58	0.000	1.63	0.002
ALDOC	Aldolase C, fructose-bisphosphate	NM_005165	−1.00	0.989	1.17	0.112
ALDOB	Aldolase B, fructose-bisphosphate	NM_000035	1.00	1.000	1.00	1.000
TPI1	Triosephosphate isomerase 1	NM_000365	1.12	0.084	**2.64**	0.000
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	NM_002046	1.45	0.011	1.76	0.010

Genes are listed in the same order of the glycolytic pathway. The expression level of genes is reported as fold change vs controls. Significant results are written in bold.

2.8. MS analysis

Spots of interest were manually excised and digestion with trypsin was performed in gel. Gel plugs were washed with acetonitrile for 10 min, dried under vacuum and reswollen using 10 μL of sequencing grade modified trypsin (Promega, Madison, WI, USA) (12.5 ng/μL in 100 mM ammonium bicarbonate). Digestion was carried out overnight at 37 °C. After digestion, the peptides were extracted by 3 changes of 50% acetonitrile/0.1% formic acid (20 min between changes), dried under vacuum, resuspended with 10 μL of 0.1% formic acid and desalted using C18 ZipTip (Millipore, Billerica, MA). The digested proteins were analyzed by MALDI-TOF (Time of Flight) MS using a M@ldi-HT (Waters, Manchester, UK). Typically, 200 shots were collected from each spot in data-dependent mode. The analyses were conducted using α-cyano-4-hydroxycinnamic acid (2.5 mg/mL in acetonitrile/0.1% formic acid 50/50) as matrix, mixing equal volumes of sample and matrix and spotting 1 μL of the mixture on a standard 96-well stainless steel MALDI target plate. The spectra were analyzed using Mascot engine search (Matrix Science, London, UK) and PIUMS® (www.hh.se/staff/bioinf). The search was performed against the human session of the IPI database (http://www.ebi.ac.uk/IPI/, version v3.22). Enzyme specificity was set to trypsin with one missed cleavage using a mass tolerance window of 50 ppm, carbamidomethylcysteine as fixed modification and oxidation of methionine as variable modification. The proteins were considered correctly identified both when the software yielded the same identification with a p value <0.05, and when the coverage of the sequence was at least 30%.

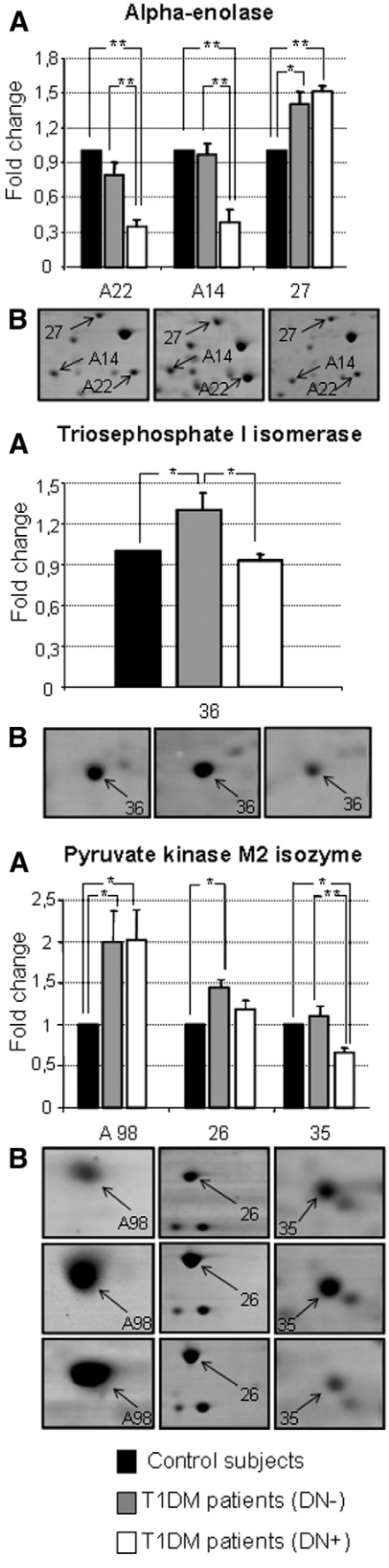
2.9. Pyruvate kinase activity

At confluence, the fibroblast cell cultures were shifted to quiescent medium for 24 h, then the medium was removed and cell monolayer washed with PBS. Cells were collected using 250 μL of 100 mM triethanolamine, 0.5 mM EDTA/Na^+ buffer (pH 7.6), supplemented with protease inhibitors and lysed by sonication. After centrifugation at 14,000 ×g for 15 min (4 °C), the surpernatant was used for protein determination [12] and enzyme assay. Pyruvate kinase (PK, EC.2.7.1.40) activity was estimated by a modification of the spectrometric method developed by Gutmann and Bernt [15]. PK activity was measured as the change in absorption of NADH at 340 nm (25 °C) due to the coupled conversion of pyruvate to lactate catalyzed by lactate dehydrogenase (EC.1.1.1.27; LDH). The reaction solution contained 97.5 mM triethanolamine pH 7.5, 13 mM $MgSO_4$, 74 mM KCl, 185 μM NADH, 1 mM PEP, 2.5 U/mL LDH, and 3 mM ADP. The enzymatic assays were repeated in

Table 2
Proteins involved in glycolysis isolated from cell cultures of fibroblasts from normal subjects and diabetic patients and identified by MS

Spot no.		Protein name	Gene name	SwissProt Accession no.	Mascot Score	*p* value	% Coverage	Matched peptides
A14	X	Alpha-enolase	ENO1	P06733	110	6.8e−7	33	11
A22	X	Alpha-enolase	ENO1	P06733	131	5.4e−9	39	13
A88		Alpha-enolase	ENO1	P06733	136	1.7e−9	65	16
13		Alpha-enolase	ENO1	P06733	147	1.4e−10	39	13
27	X	Alpha-enolase	ENO1	P06733	122	4.3e−8	39	14
83		Fructose-bisphosphate aldolase A	ALDOA	P04075	297	1.4e−25	75	25
4		Glyceraldehyde-3-Phosphate Dehydrogenase	GAPDH	Q5D0F4	115	2.1e−7	43	9
A89		Phosphoglycerate kinase 1	PGK1	P00558	116	1.7e−7	43	14
A98	X	Pyruvate kinase, isozymes M1/M2	PKM2	P14618-2	135	2.1e−9	45	21
26	X	Pyruvate kinase, isozymes M1/M2	PKM2	P14618-2	121	5.4e−8	30	12
35	X	Pyruvate kinase, isozymes M1/M2	PKM2	P14618-2	126	1.7e−8	31	15
60		Pyruvate kinase, isozymes M1/M2	PKM2	P14618-2	247	1.4e−20	42	23
61		Pyruvate kinase, isozymes M1/M2	PKM2	P14618-2	108	1.1e−6	30	12
66		Pyruvate kinase, isozymes M1/M2	PKM2	P14618-2	180	6.8e−14	34	17
A82		Pyruvate kinase, isozymes M1/M2	PKM2	P14618-2	99	8.7e−6	30	14
36	X	Triosephosphate Isomerase	TPI1	P60174	180	6.8e−14	75	14
57		Triosephosphate Isomerase	TPI1	P60174	177	1.4e−13	72	17

The gene names and the accession numbers are referred to the human session of the SwissProt/TrEMBL database (http://www.expasy.org/). Proteins labelled with an X correspond to proteins significantly altered among groups. Mascot score, p value, % of sequence coverage and number of matched peptides are also reported.



triplicate for each subject, and the mean was used in the statistical analysis.

2.10. Statistical analysis

Cluster analysis was performed to determine similarities (or differences) among the arrays and/or sequences. A cut-off value of 2-fold was used to identify both over- and under-expressed genes. Differentially abundant protein spots and PK activity among groups were analysed using one-way ANOVA and the post hoc Bonferroni testing (Statistica, StatSoft Italia srl). A p value less than 0.05 was considered statistically significant. Results were expressed as mean and standard error.

3. Results

3.1. Microarray analysis

The transcriptomic analysis of glycolytic enzymes did not show any significant difference between T1DM(DN−) group and control subjects. In T1DM(DN+) patients, compared to control subjects, we found an over-expression of hexokinase 2 (HK2), phosphoglucomutase 1 (PGM1), phosphofructokinase (PFKL, transcript variant 1), aldolase A (ALDOA, transcript variant 1) and triosephosphate isomerase-1 (TPI-1) (Table 1).

3.2. 2-DE analysis

We identified a total of 17 spots belonging to proteins involved in glycolysis (Table 2). This group accounted for 12.5% of all the proteins isolated and characterized by our group in cultured skin fibroblasts from both T1DM patients and normal subjects [15]. Because of the alternative splicing of mRNA transcripts and/or post-translational modifications, some of these spots likely represent different isoforms of the same protein. In all, six unique proteins were identified (according to Human Protein Reference Database, www.hpdr.org) (Table 2). As shown in Fig. 1, significant differences were detected among the three groups of subjects in the abundance of 7 spots, corresponding to 3 unique proteins (alpha-enolase, triosephosphate I isomerase, and pyruvate kinase).

3.3. Pyruvate kinase activity

For the three glycolytic enzyme–proteins, whose expressions were altered in the diabetic groups, an enzymatic activity assay could be performed only for PK, because the reagents to measure either alpha-enolase or TPI activity were not available. A significant reduction (by≈40%, $p<0.01$) in PK activity was detected in fibroblasts from T1DM(DN+) patients relative to the fibroblasts from the two other groups (Fig. 2).

4. Discussion

Type 1 diabetic patients with DN exhibit peripheral insulin resistance to glucose utilization [3], and the pathogenesis of DN has been linked to an altered intracellular glucose metabolism [16]. Nevertheless, the site(s) and the mechanism(s) of insulin resistance in T1DM(DN+) patients is poorly understood. In this study, we examined the expression of glycolytic enzymes both at the transcriptome and at the proteome level in cultured skin fibroblasts from T1DM(DN+) and (DN−) patients, as well as in matched control

Fig. 1. Quantitative analysis of protein changes in skin fibroblasts from control subjects and diabetic patients. (A) Volume density analysis graphs: the data (means±SEM) are expressed as fold changes vs. controls (*$p<0.05$; **$p<0.01$); B) 2-DE gel images of selected spots.

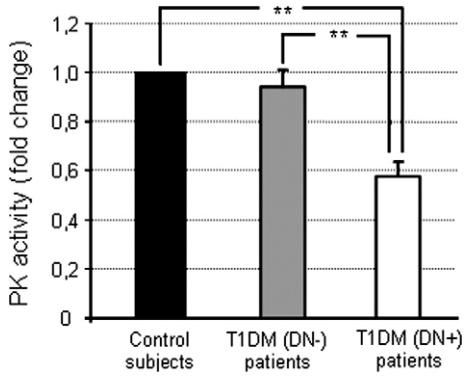


Fig. 2. Pyruvate kinase activity in cultured skin fibroblasts from T1DM patients with and without DN and normal control subjects. Data (means±SEM) are expressed as fold changes vs. controls (**$p<0.01$).

subjects. Additionally, we measured cell pyruvate kinase activity under the same conditions.

Based on the transcriptome data in fibroblasts from T1DM(DN+) patients, we found an over-expression of several enzymes involved in the glycolytic cascade (HK2, PGM1, PFKL, ALDOA, and TPI-1) (Table 1). At the proteomic level, we observed alterations in the expression of TPI, PK and alpha-enolase (Fig. 1). A reduction in PK activity was also observed in fibroblasts from diabetic patients with DN (Fig. 2). While these data may indicate novel sites for insulin resistance in T1DM(DN+) patients, their interpretation is complex as there was little correlation between the transcriptomic and the proteomic profiles. Thus, it appears that transcriptional control has little effect on the quantitative changes of these proteins in the studied groups, and that the expression of glycolytic proteins is instead regulated at a post-transcriptional level.

Classic sites of insulin resistance in the glycolytic cascade have been identified at the level of glucose transport, phosphorylation and glycogen synthesis [17,18], glyceraldehyde-3-phosphate dehydrogenase (GA3PDH) [19], the pentose cycle [19], the hexosamine pathway [20], the pyruvate dehydrogenase complex [21], and glucose oxidation [22]. The final reaction of glycolysis is catalyzed by the tightly regulated enzyme pyruvate kinase, which converts phosphoenolpyruvate (PEP) to pyruvate. In this highly exergonic reaction, the high-energy phosphate of PEP is conserved as ATP. Here we discuss the glycolytic sites which were altered in the diabetic patients with nephropathy.

4.1. Pyruvate kinase (EC 2.7.1.40)

From the literature, there is conflicting data on PK in diabetes. PK activity and mRNA expression were found to be decreased both in adipose tissue [23] and in cultured pancreatic islets [24] of T1DM patients, as well as in animal models of insulin-deficient diabetes [25]. However, PK activities that were only slightly increased [26] or virtually unchanged [27] were also reported. These observations are particularly important since it is well established that insulin activates PK [28].

In fibroblasts from diabetic patients, we observed increases in the expression of the #A98 isoform (in both diabetic groups) and of the #26 isoform (only in the T1DM(DN−) group) of PK compared to controls, whereas the #35 isoform expression was specifically decreased in fibroblasts from T1DM(DN+) patients. An overall decrease in PK activity in fibroblasts from T1DM(DN+) patients was observed. Since the assay of enzymatic activity cannot distinguish between the activities of each individual isoform, we cannot determine whether the decreased expression of the #35 isoform, an altered structure of the other two isoforms, or both factors working concomitantly, were responsible for the overall decrease of PK activity. Nevertheless, our data suggest a shift in the enzyme isoforms, a hypothesis supported also by the invariant PK mRNA expression among groups (Table 1). Further studies of PK structure, with specific regard to possible post-translational modifications, will hopefully clarify these points. Insulin stimulates the expression of the M-PK gene in 3T3-L1 adipocytes, through both PI3 and MAP kinases [29]. In insulin-resistant conditions, insulin action on the PI3K intracellular signalling pathway is impaired [30], whereas signalling through the extracellular signal-regulated kinase MAPK pathway is unaffected [31]. Thus, from our data, it could be argued that PK activity is predominantly activated through the PI3K pathway, and that a reduction in PK activity in T1DM(DN+) patients could be responsible for insulin resistance at a distal site along the glycolytic cascade.

4.2. Triosephosphate I isomerase (EC 5.3.1.1)

We did not find any correlation between the RNA expression and the protein level of TPI among groups. In fact, as reported in Table 1 and Fig. 1, the TPI gene expression in fibroblasts from T1DM (DN+) patients was increased, while the protein abundance was similar to that of controls. On the contrary, in fibroblasts from T1DM(DN−) patients, TPI gene expression was similar to that of controls, while the protein abundance (spot #36) was increased with respect to that of the other groups. In the T1DM(DN+) subjects there was an increase in TPI gene expression which did not correlate with an increase in the protein level, probably due to post-transcriptional processing. These data further indicate that transcriptional control might have little effect on the abundance changes of this protein.

TPI catalyses the interconversion of di-hydroxyacetone phosphate to D-glyceraldehyde 3-phosphate (GAP). GAP is then further oxidized to 1,3 diphosphoglycerate by GAP-dehydrogenase (GAPDH). Du et al. [32] reported that GAPDH activity is reduced in T1DM, both in humans and in animal models, as well as in cells exposed to high glucose. Decreased GAPDH activity leads to increased glycolytic intermediates upstream of this enzymatic step [33], which subsequently leads to activation of two other pathways which are altered in diabetes (i.e. the advanced glycosylation end-product pathway and the PKC pathway). Under normal conditions, even if the concentration of GAP is lower than that of its isomer di-hydroxyacetone phosphate (<10% vs. >90% respectively) [34], a decrease in GAP oxidation could increase its concentration, and this may allosterically activate TPI and enhance GAP disposal through an alternative pathway. Our data indirectly suggest that in DN, thereis a lesser activation of the TPI and thus a possible decrease in the interconversion of di-hydroxyacetone phosphate to GAP.

4.3. Alpha-enolase (EC 4.2.1.11)

A decreased expression of two isoforms of alpha-enolase (#A22, #A14) was detected in the diabetic subjects with renal complications (Fig. 2). Alpha-enolase (an enolase isoenzyme), is a key protein that catalyzes the conversion of 2-phosphoglycerate to phosphoenolpyruvate and plays a crucial role in the regulation of glycolysis, as well as in other processes such as growth control, hypoxia tolerance, and allergic responses [35]. In aged monkeys, the expression of alpha-enolase in the heart and of other proteins involved in energy metabolism was decreased, suggesting an age-related mechanism within this tissue [36]. Furthermore, in systemic autoimmune diseases, autoantibodies specific for alpha-enolase are detected more frequently in patients with active renal involvement [37,38]. These antibodies recognize the membrane-associated form and/or interfere with the receptor function of this enzyme, thus inhibiting the binding of plasminogen activator (PA). To our

knowledge, there has been no report on the prevalence of alpha-enolase autoantibodies in type 1 diabetes, which is also considered an autoimmune disease. It could be interesting to study this important issue in more depth. One of the critical responses to vascular injury is the activation of the PA system, including both tissue-type and urinary-type plasminogen activator, which converts PA to plasmin. Plasmin, in turn, degrades fibrin and several extracellular matrix proteins. Fibrin deposition is an important factor in the development of vascular disorders such as atherosclerosis, and the accumulation of extracellular matrix proteins is a hallmark of renal fibrosis and of the resultant albuminuria. PA inhibition, possibly due to an alteration of enolase binding (in turn due to either altered expression or post-translational modification of this enzyme) could lead to an accumulation of extracellular matrix proteins, as is commonly observed in DN.

The expression of the #27 alpha-enolase isoform was increased in the diabetic groups compared to normal subjects (Fig. 2). Conversely, the abundance of the two isoforms #A22 and #A14 was decreased only in fibroblasts from T1DM(DN+) patients.

The likelihood of post-translational modifications of this enzyme is supported by the finding that enolase gene expression is not significantly different among groups (Table 1). Our data are the first to show an association between alpha-enolase isoform alterations and DN.

Since the fibroblasts were studied under euglycemic conditions after standard repeated passages, any effect of the differential ambient glucose concentrations and of other conditioning factors (like drugs, inflammatory conditions, etc) between the diabetic and control subjects has been excluded.

However, the effects of previous long-term hyperglycemia, resulting in persistent changes in PK activity in the diabetic subjects, cannot strictly be ruled out. Since the long-term metabolic control in the two groups of T1DM patients was similar, as indicated by the HbA1c level (see Materials and methods), the differences observed between the two diabetic groups cannot be attributed to differences in the previous glucose levels.

In conclusion, we have reported altered expression and activity of some glycolytic enzymes in cultured skin fibroblasts from T1DM(DN+) patients. These novel alterations could be responsible for the insulin resistance found in these conditions and be associated with the pathogenesis of diabetic nephropathy.

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References

[1] M. Krolewski, P.W. Eggers, J.H. Warram, Magnitude of end-stage renal disease in IDDM: a 35-year follow-up study, Kidney Int. 50 (1996) 2041–2046.
[2] J. Yip, M.B. Mattock, A. Morocutti, M. Sethi, R. Trevisan, G. Viberti, Insulin resistance in insulin-dependent diabetic patients with microalbuminuria, Lancet 342 (1993) 883–887.
[3] P.H. Groop, C. Forsblom, M.C. Thomas, Mechanisms of disease: pathway-selective insulin resistance and microvascular complications of diabetes, Nat. Clin. Pract. Endocrinol. Metab. 1 (2005) 100–110.
[4] S. De Cosmo, A. Argiolas, G. Miscio, S. Thomas, G.P. Piras, R. Trevisan, P.C. Perin, S. Bacci, L. Zucaro, M. Margaglione, L. Frittitta, A. Pizzuti, V. Tassi, G.C. Viberti, V. Trischitta, A PC-1 amino acid variant (K121Q) is associated with faster progression of renal disease in patients with type 1 diabetes and albuminuria, Diabetes 49 (2000) 521–524.
[5] E. Iori, M.C. Marescotti, M. Vedovato, G. Ceolotto, A. Avogaro, A. Tiengo, S. Del Prato, R. Trevisan, In situ protein kinase C activity is increased in cultured fibroblasts from Type 1 diabetic patients with nephropathy, Diabetologia 46 (2003) 524–530.
[6] M.S. LaPointe, D. Batlle, Cultured skin fibroblasts as an in vitro model to assess phenotypic features in subjects with diabetic nephropathy, Am. J. Kidney Dis. 38 (2001) 646–648.
[7] M.S. Simonson, Phenotypic transitions and fibrosis in diabetic nephropathy, Kidney Int. 71 (2007) 846–854.
[8] J.E. Pessin, S. Okada, Insulin and EGF receptors integrate the Ras and Rap signaling pathways, Endocr. J. 46 (1999) S11–S16.
[9] F. Podestà, G. Meregalli, R. Ghelardi, R. Del Giudice, V. Asnaghi, A. Maestroni, G. Zerbini, Low Ca(2+) pump activity in diabetic nephropathy, Am. J. Kidney Dis. 38 (2001) 465–472.
[10] N.A. Shackel, D. Seth, P.S. Haber, M.D. Gorrell, G.W. McCaughan, The hepatic transcriptome in human liver disease, Comp. Hepatol. 5 (2006) 6.
[11] T.J. Griffin, S.P. Gygi, T. Ideker, B. Rist, J. Eng, L. Hood, R. Aebersold, Complementary profiling of gene expression at the transcriptome and proteome levels in Saccharomyces cerevisiae, Mol. Cell. Proteomics 4 (2002) 323–333.
[12] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.
[13] L. Lenzini, T.M. Seccia, E. Alighieri, A.S. Belloni, P. Bernante, L. Giuliani, G.G. Nussdorfer, A.C. Pessina, G.P. Rossi, Heterogeneity of aldosterone-producing adenomas revealed by a whole transcriptome analysis, Hypertension 50 (2007) 1106–1113.
[14] L. Puricelli, E. Iori, R. Millioni, G. Arrigoni, P. James, M. Vedovato, P. Tessari, Proteome analysis of cultured fibroblasts from type 1 diabetic patients and normal subjects, J. Clin. Endocrinol. Metab. 91 (2006) 3507–3514.
[15] I. Gutmann, E. Bernt, Pyruvate kinase, in: HU Bergmeyer (Ed.), Methods of Enzymatic Analysis, 2, Verlag Chemie Weinheim, Academic Press, Inc., New York and London, 1974, pp. 774–777.
[16] C. Meyer, A. Tolias, D. Platanisiotis, M. Stumvoll, L. Vlachos, A. Mitrakou, Increased renal glucose metabolism in Type 1 diabetes mellitus, Diabet. Med. 22 (2005) 453–459.
[17] H. Beck-Nielsen, Mechanisms of insulin resistance in non-oxidative glucose metabolism: the role of glycogen synthase, J. Basic Clin. Physiol. Pharmacol. 9 (1998) 255–279.
[18] M. Ristow, M. Vorgerd, M. Möhlig, H. Schatz, A. Pfeiffer, Deficiency of phosphofructo-1-kinase/muscle subtype in humans impairs insulin secretion and causes insulin resistance, J. Clin. Invest. 100 (1997) 2833–2841.
[19] F. Leyva, C.S. Wingrove, I.F. Godsland, J.C. Stevenson, The glycolytic pathway to coronary heart disease: a hypothesis, Metabolism 47 (1998) 657–662.
[20] M.G. Buse, Hexosamines, insulin resistance, and the complications of diabetes: current status, Am. J. Physiol. Endocrinol. Metab. 290 (2006) E1–E8.
[21] M.J. Holness, M.C. Sugden, Regulation of pyruvate dehydrogenase complex activity by reversible phosphorylation, Biochem. Soc. Trans. 31 (2003) 1143–1151.
[22] M.E. Patti, A.J. Butte, S. Crunkhorn, K. Cusi, R. Berria, S. Kashyap, Y. Miyazaki, I. Kohane, M. Costello, R. Saccone, E.J. Landaker, A.B. Goldfine, E. Mun, R. DeFronzo, J. Finlayson, C.R. Kahn, L.J. Mandarino, Coordinated reduction of genes of oxidative metabolism in humans with insulin resistance and diabetes: potential role of PGC1 and NRF1, Proc. Natl. Acad. Sci. U. S. A. 100 (2003) 8466–8471.
[23] F. Belfiore, A.M. Rabuazzo, E. Napoli, V. Borzi, L. Lo Vecchio, Enzymes of glucose metabolism and of the citrate cleavage pathway in adipose tissue of normal and diabetic subjects, Diabetes 24 (1975) 865–873.
[24] R. Lupi, L. Marselli, S. Dionisi, S. Del Guerra, U. Boggi, M. Del Chiaro, C. Lencioni, M. Bugliani, F. Mosca, U. Di Mario, S. Del Prato, F. Dotta, P. Marchetti, Improved insulin secretory function and reduced chemotactic properties after tissue culture of islets from type 1 diabetic patients, Diabetes Metab. Res. Rev. 20 (2004) 246–251.
[25] Y. Kondoh, M. Kawase, Y. Kawakami, S. Ohmori, Concentrations of D-lactate and its related metabolic intermediates in liver, blood, and muscle of diabetic and starved rats, Res. Exp. Med. (Berl) 192 (1992) 407–414.
[26] Y.Z. Diamant, E. Shafrir, Placental enzymes of glycolysis, gluconeogenesis and lipogenesis in the diabetic rat and in starvation. Comparison with maternal and foetal liver, Diabetologia 15 (1978) 481–485.
[27] I. Rossi, J.A. Sanchez-Arias, J.E. Feliu, Effect of streptozotocin diabetes on the glycolytic flux and on fructose 2,6-bisphosphate levels in isolated rat enterocytes, Metabolism 39 (1990) 882–885.
[28] W.C. Parks, R.L. Drake, Insulin mediates the stimulation of pyruvate kinase by a dual mechanism, Biochem. J. 208 (1982) 333–337.
[29] Y. Asai, K. Yamada, T. Watanabe, V.W. Keng, T. Noguchi, Insulin stimulates expression of the pyruvate kinase M gene in 3T3-L1 adipocytes, Biosci. Biotechnol. Biochem. 67 (2003) 1272–1277.
[30] J.E. Pessin, A.R. Saltiel, Signaling pathways in insulin action: molecular targets of insulin resistance, J. Clin. Invest. 106 (2000) 165–169.
[31] K. Cusi, K. Maezono, A. Osman, M. Pendergrass, M.E. Patti, T. Pratipanawatr, R.A. DeFronzo, C.R. Kahn, L.J. Mandarino, Insulin resistance differentially affects the PI 3-kinase- and MAP kinase-mediated signalling in human muscle, J. Clin. Invest. 105 (2000) 311–320.
[32] X. Du, T. Matsumura, D. Edelstein, L. Rossetti, Z. Zsengeller, C. Szabo, M. Brownlee, Inhibition of GAPDH activity by poly(ADP-ribose) polymerase activates three major pathways of hyperglycemic damage in endothelial cells, J. Clin. Invest. 112 (2003) 1049–1057.
[33] M. Brownlee, The pathobiology of diabetic complications: a unifying mechanism, Diabetes 54 (2005) 1615–1625.
[34] A.L. Lehninger, Biochemistry, Worth Publisher Inc., New York, USA, 1970, p. 321.

[35] V. Pancholi, Multifunctional alpha-enolase: its role in diseases, Cell. Mol. Life Sci. 58 (2001) 902–920.
[36] L. Yan, H. Ge, H. Li, S.C. Lieber, F. Natividad, R.R. Resuello, S.J. Kim, S. Akeju, A. Sun, K. Loo, A.P. Peppas, F. Rossi, E.D. Lewandowski, A.P. Thomas, S.F. Vatner, D.E. Vatner, Gender-specific proteomic alterations in glycolytic and mitochondrial pathways in aging monkey hearts, J. Mol. Cell. Cardiol. 37 (2004) 921–929.
[37] N. Akisawa, T. Maeda, S. Iwasaki, S. Onishi, Identification of an autoantibody against alpha-enolase in primary biliary cirrhosis, J. Hepatology 26 (1997) 845–851.
[38] T. Orth, R. Kellner, O. Diekmann, J. Faus, K.H. Meyer zum Büschenfelde, W.J. Mayet, Identification and characterization of autoantibodies against catalase and alphaenolase in patients with primary sclerosing cholangitis, Clin. Exp. Immunol. 112 (1998) 507–515.