High glucose repatterns human podocyte energy metabolism during differentiation and diabetic nephropathy

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ABSTRACT: Podocytes play a key role in diabetic nephropathy pathogenesis, but alteration of their metabolism remains unknown in human kidney. By using a conditionally differentiating human podocyte cell line, we addressed the functional and molecular changes in podocyte energetics during in vitro development or under high glucose conditions. In 5 mM glucose medium, we observed a stepwise activation of oxidative metabolism during cell differentiation that was characterized by peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α)–dependent stimulation of mitochondrial biogenesis and function, with concomitant reduction of the glycolytic enzyme content. Conversely, when podocytes were cultured in high glucose (20 mM), stepwise oxidative phosphorylation biogenesis was aborted, and a glycolytic switch occurred, with consecutive lactic acidosis. Expression of the master regulators of oxidative metabolism transcription factor A mitochondrial, PGC-1α, AMPK, and serine–threonine liver kinase B1 was altered by high glucose, as well as their downstream signaling networks. Focused transcriptomics revealed that myocyte-specific enhancer factor 2C (MEF2C) and myogenic factor 5 (MYF5) expression was inhibited by high glucose levels, and endoribonuclease-prepared small interfering RNA–mediated combined inhibition of those transcription factors phenocopied the glycolytic shift that was observed in high glucose conditions. Accordingly, a reduced expression of MEF2C, MYF5, and PGC-1α was found in kidney tissue sections that were obtained from patients with diabetic nephropathy. These findings obtained in human samples demonstrate that MEF2C–MYF5–dependent bioenergetic dedifferentiation occurs in podocytes that are confronted with a high-glucose milieu.—Imasawa, T., Obre, E., Bellance, N., Lavie, J., Imasawa, T., Rigothier, C., Delmas, Y., Combe, C., Lacome, D., Benard, G., Claverol, S., Bonneu, M., Rossignol, R. High glucose repatterns human podocyte energy metabolism during differentiation and diabetic nephropathy. FASEB J. 31, 294–307 (2017). www.fasebj.org

KEY WORDS: mitochondria · MEF2C · human kidney

ABBREVIATIONS: esiRNA, endoribonuclease-prepared small interfering RNA; LKB1, serine–threonine liver kinase B1; MEF2C, myocyte-specific enhancer factor 2C; mtDNA, mitochondrial DNA; mTOR, mammalian target of rapamycin; MYF5, myogenic factor 5; NRF-1, nuclear respiratory factor-1; oxphos, oxidative phosphorylation; PDK1, pyruvate dehydrogenase kinase 1; PGC-1α, peroxisome proliferator-activated receptor-γ coactivator 1α; PKM2, pyruvate kinase muscle type 2; TCA, tricarboxylic acid; TFAM, transcription factor A, mitochondrial

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One in 2 patients with diabetes suffers from diabetic nephropathy, the leading cause of chronic kidney disease worldwide (1). Previous studies have revealed that glomerular visceral epithelial cells, namely podocytes, could play a key role in the pathogenesis of diabetic nephropathy (2–5). Podocytes have complex structures, which are known as foot processes, slit diaphragm, and the focal adhesion complex, all of which are altered in diabetic nephropathy (2–5).

Because a considerable number of mitochondria have been observed in the cell body and in the narrow peripheral foot processes, it is thought that podocytes could be sensitive to an alteration of oxidative metabolism (6, 7). Furthermore, in cases of mitochondrial disorders, podocytes lose the interdigitating foot processes and accumulate dysmorphic mitochondria (8, 9). Such foot process effacement resembles an early morphologic change of
diabetic nephropathy (2); therefore, understanding the peculiarities of podocyte energy metabolism, notably in diabetic conditions, could provide novel insights into the pathogenesis of diabetic nephropathy and the role of mitochondria (10, 11).

We hypothesized that high glucose levels could alter mitochondrial function in human podocytes, as found in pancreatic β-cells (12), muscle cells (13), adipocytes (14), or neurons (15), which should lead to diabetic complications (16). Because podocytes dedifferentiate in the process of injuries as diabetic nephropathy (17, 18), we investigated podocyte energetics and proteome remodeling during normal differentiation as well as under hyperglycemic stress.

**MATERIALS AND METHODS**

**Cell culture**

Human podocytes, previously described (19), were used in our study. These cells began to differentiate after switching from 33°C to 37°C and were fully differentiated 15 d after this switch (19, 20). Podocytes were cultured in RPMI 1640 medium that was supplemented with 10% fetal calf serum, insulin (10 μg/ml; Sigma-Aldrich, Lyon, France), transferrin (5.5 μg/ml; Sigma-Aldrich), penicillin-streptomycin 1X (100×; Thermo Fisher Scientific, Waltham, MA, USA), with 5 mM glucose from d 0 (day of temperature switch) to d 7 (d after temperature switch). We confirmed that podocytes at d 15 expressed nephrin, podocin, and podocalyxin by Western blot. To observe mitochondrial network morphology, MitoTracker Red CMXRos 100 nM (Thermo Fisher Scientific) was added to culture medium and cells were incubated at 37°C for 25 min (21).

**Cellular oxygen consumption rate**

Endogenous cellular oxygen consumption rate was monitored in intact podocytes at 37°C in a 1-ml thermostatically controlled chamber (1.0 × 10⁶ cells/ml/run) that was equipped with a Clark oxygen electrode (Hansatech Instruments Ltd., Norfolk, United Kingdom). Endogenous respiratory rate was expressed in nanomolar O₂ per min per 10⁶ cells. Data were collected from 3 independent flasks for each condition.

**Cellular and mitochondrial ATP synthesis**

Podocytes were collected and resuspended in culture medium at a concentration of 5 × 10⁵ cells/ml. Cell suspension (0.1 ml/tube) was incubated at 37°C for 45 min with or without 5 μl of 100 mM antimycin (Sigma-Aldrich) to block mitochondrial respiration and ATP synthesis. After incubation, cells were treated with a cell lysis buffer (ATP Bioluminescence Assay Kit CLS II; Roche, Basel, Switzerland) and ATP content that was released into the medium was measured by bioluminescence (21). ATP synthesis was measured in the presence of antimycin.

**Mitochondrial biogenesis evaluation**

We measured the mitochondrial DNA (mtDNA) content and mRNA levels of peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α). After collecting cultured podocytes, total DNA was extracted by using a DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA, USA), and total RNA was extracted by using an RNeasy Plus Mini Kit (Qiagen). First-strand cDNA was synthesized in a 20-μl volume using 1 μg total RNA, with 50 units of M-MuLV Reverse Transcriptase (RNase H⁻) and Oligo d(T)₁₆ (Applied Biosystems, Foster City, CA, USA). Real-time quantitative PCR for nuclear DNA, mtDNA, and PGC-1α mRNA was performed by using iQSYBR Green Supermix (Bio-Rad, Hercules, CA, USA) on a thermocycler CFX96 Touch Real-Time PCR system (Bio-Rad), as previously described (22). Normalized units to the endogenous references (RPLP0 and GUSB) were calculated by using the CFX Manager Software program (Bio-Rad).

**Measurement of enzymatic activities of respiratory chain complexes and citrate synthase**

All assays were performed in 200 μl of Tp III mannitol buffer (225 mM mannitol, 75 mM saccharose, 10 mM TrisHCl, 0.1 mM EDTA, pH 7.2). All results were normalized by protein concentration (BCA protein assay; Pierce, Rockford, IL, USA). We determined several respiratory chain complexes (complex III and IV) and citrate synthase enzymatic activities, as previously described (23).

**Western blotting**

Western blot was performed as previously described (22) by using the following antibodies: PGC-1α (P-19; sc-5815, 1:100; Santa Cruz Biotechnology; Santa Cruz, CA, USA), nuclear respiratory factor-1 (NRF-1; ab55744, 1:500; Abcam, Cambridge, United Kingdom), transcription factor A, mitochondrial (TFAM; ab119684, 1:500; Abcam), AMPK (2532, 1:1000; Cell Signaling Technology, Danvers, MA, USA), phospho-AMPK-α for the detection of endogenous AMPKα only when phosphorylated at threonine 172 (2531, 1:1000; Cell Signaling Technology), siren–threonine liver kinase B1 (LKB1; ab15095, 1:100; Abcam), and mammalian target of rapamycin (mTOR; ab87540, 1:200; Abcam).

**Reactive oxygen species measurement**

Changes in reactive oxygen species levels were monitored by using the CM-H₂DCFDA probe (C6827; Thermo Fisher Scientific). Cells were plated in 96 wells (20,000 cells/well). The probe was added to cell medium and incubated for 30 min at 37°C. Of H₂O₂, 10 μl was used a positive control. Fluorescence was measured on a Xenius Spectrofluorometer (Safas, Monaco, France).

**Label-free quantitative proteomics**

This analysis was performed by the proteomic facility of Bordeaux University (http://www.ecbf.u-bordeaux2.fr/en/synthese-proteome). The steps of sample preparation and protein digestion, nano-scale liquid chromatography–tandem mass spectrometry analysis, and database search and results processing were performed as previously described (24). Label-free quantitative data analysis was performed by using Progenesis Liquid Chromatography–Tandem Mass Spectrometry 4.0 software (Nonlinear Dynamics, Newcastle, United Kingdom). Data processing included the following steps: 1) feature detection, 2) feature alignment, 3) volume integration for 2-6 charge-state
Figure 1. Podocyte physiology and energetics during differentiation. A) Representative images of cultured, differentiating podocytes at the day of switching to 37°C (d 0), at 3 d after the switch (d 3), and at d 7, 11, and 15 (mature podocytes). The mitochondrial network (white tubules) was stained with MitotrackerGreen. B) Oxygen consumption rate (nmol/min/×10^5 cells) at each differentiation stage. C) Changes of cellular ATP content (pmol/×10^5 cells) during differentiation. D) Changes of ATP content produced by oxphos (pmol/×10^5 cells) during differentiation. E) Changes of ATP content produced by glycolysis (pmol/×10^5 cells) during differentiation. (continued on next page)
ions, 4) normalization of the total protein abundance, 5) import of sequence information, 6) ANOVA test at the peptide level and filtering for features \( P < 0.05 \), 7) calculation of protein abundance (sum of the volume of corresponding peptides), and 8) ANOVA test at the protein level and filtering for features \( P < 0.05 \). Of note, only nonconflicting features and unique peptides were considered for calculation at the protein level. We selected proteins that showed \( >20\% \) change in their expression levels with statistical significance between 2 groups (\( n = 3 \) in each group). Furthermore, these proteins were categorized according to their functions by using the Kyoto Encyclopedia of Genes and Genomes analysis in the search tool for the retrieval of interacting genes/proteins (STRING) database (http://string-db.org). A more global analysis of the data was performed via use of Ingenuity Pathway Analysis (Qiagen).

### Results

**Analysis by quantitative PCR microarray**

In this study, we used 2 different quantitative PCR microarrays: Human Glucose Metabolism RT² Profiler PCR Array (Qiagen) and Human Transcription Factors RT² Profiler PCR Array (Qiagen).

**Analyses by endoribonuclease-prepared small interfering RNA**

At d 12, we transfected cells with endoribonuclease-prepared small interfering RNA (esiRNA) under 4 conditions: esiRNA enhanced green fluorescent protein, esiRNA myogenic factor 5 (MYF5), esiRNA myocyte-specific enhancer factor 2C (MEF2C), and esiRNA MYF5 plus MEF2C. Human esiRNA from Sigma-Aldrich and Dharmacon RNA Technologies (Lafayette, CO, USA) were used. We prepared 5 \( \mu \)M esiRNA solutions in serum-free medium, mixed them with Dharmafect transfection reagent provided by Dharmacon RNA Technologies (Lafayette, CO, USA) and incubated them at room temperature for 20 min. Then, antibiotic-free complete medium was added for a final esiRNA concentration of 25 nM. We removed the medium in flasks and added the appropriate esiRNA solution for each condition. Podocytes were cultured in 5 mM glucose. At d 15, protein from podocytes was extracted.

**Analyses of human kidney samples**

Kidney biopsied tissues from 5 patients with diabetic nephropathy and 5 normal participants were used in this study. Primary antibodies were PGC-1\( \alpha \) (P-19; sc-5815; Santa Cruz Biotechnology) and pyruvate kinase muscle type 2 (PKM2; D78A4; #4053; Cell Signaling Technology), as well as MYF5 (C-20, sc-302; Santa Cruz Biotechnology) and MEF2C (ab191428; Abcam). Numbers of PKM2-positive podocytes per glomerulus were counted. In addition, we calculated mean percentages of PGC-1\( \alpha \)-, MYF5-, and MEF2C-positive nuclei of total nuclei per glomerulus; more than 10 glomeruli per patient were counted in these analyses. This study was approved by the ethical committee of Chiba-East Hospital.

### RESULTS

**Activation of oxidative energy metabolism during human podocyte in vitro differentiation**

Upon induction of podocyte differentiation (19, 20), cell morphology changed from a small, circular cell to a reticulated one, which became mature at d 15. The size of the mitochondrial network also increased progressively (Fig. 1A). Oxygen consumption rate (Fig. 1B) increased significantly during cell differentiation (\( P < 0.01 \), ANOVA analysis), with an increment factor of 2.2 after 15 d of growth in 5 mM glucose medium. Cellular ATP content (Fig. 1C) also increased between d 0 and 15 (\( P < 0.05 \), ANOVA analysis), and this increase was attributed to oxidative phosphorylation (oxphos), but not glycolysis (Fig. 1D–F). Accordingly, the amount of mtDNA increased during differentiation (Fig. 1G). Likewise, PGC-1\( \alpha \) mRNA levels increased significantly (Fig. 1H), with a factor of 2.7 between d 0 and 15. In addition, NRF-1 and TFAM mRNA expression also increased by factors of 1.5 and 4.2, respectively (Fig. 1I, J).

**Molecular changes in bioenergetic machineries during human podocyte differentiation**

To investigate the molecular changes in podocytes undergoing differentiation, we compared the proteome between d 0–3, 3–7, and 7–15 (Table 1). Approximately 8\% of the proteins whose expression levels changed during differentiation. F) Contribution of oxphos to total ATP production. G) mtDNA/nDNA (nuclear DNA) ratio determined by using quantitative PCR. H) mRNA content of PGC-1\( \alpha \) measured by quantitative PCR. I) NRF-1 expression determined by Western blotting. K) TFAM expression determined by Western blotting. Histograms indicate means ± SD. \( P \) values by 1-way ANOVA are indicated in graphs.

### Table 1. Summary of results by quantitative proteomics

| Results | 0–3 d | 3–7 d | 7–15 d | NG vs. HG |
|---------|-------|-------|--------|-----------|
| Mitochondrial protein/total protein | 104/1451 (7.2) | 124/1579 (7.9) | 102/1416 (7.2) | 40/320 (7.7) |
| Increased mitochondrial protein/increased total protein | 55/549 (10.0) | 84/967 (8.7) | 61/672 (9.1) | 9/230 (3.9) |
| Decreased mitochondrial protein/decreased total protein | 49/902 (5.4) | 40/612 (6.5) | 41/744 (5.5) | 31/290 (10.7) |

Number of proteins whose expression levels changed at 3 stages of podocyte differentiation and between podocytes cultured in the normal glucose and HG media \([n/N (\%)]\). These results were obtained by using mass spectrometry-based quantitative proteomics. Only proteins that showed \( >20\% \) change in their expression levels with statistical significance between 2 groups were selected. NG, normal glucose. \( P < 0.05 \).
within those 3 periods belonged to mitochondria. Among proteins that increased between d 0–3 and 3–7 (Fig. 2A, B), the proteasome comprised the largest population (>30 proteins). Then, >10 proteins associated with glycolysis/gluconeogenesis, oxphos, and the tricarboxylic acid (TCA) cycle also increased during both periods. From d 7–15 (Fig. 2C), a considerable number of proteins (>40) that were associated with ribosome biogenesis and...
| Changes of energy metabolism in podocytes | Stage (d) | | | |
|-----------------------------------------|-----------|-----------|-----------|-----------|
| Krebs cycle                             | 0–3       | 3–7       | 7–15      | HG/NG     |
| Pyruvate dehydrogenase E1 component subunit β | –         | –         | –         | uc        |
| Isocitrate dehydrogenase                | ++        | ++        | ++        | uc        |
| Aconitate hydratase                     | uc uc uc uc | uc uc uc uc | uc uc uc uc | uc uc uc uc |
| Complex I                               |           |           |           |           |
| NADH-ubiquinone oxidoreductase 75-kDa subunit | +++       | +         | –         | uc        |
| NADH dehydrogenase iron-sulfur protein 2 | ++        | uc uc uc uc | uc uc uc uc | uc uc uc uc |
| NADH dehydrogenase iron-sulfur protein 3 | uc uc uc uc | uc uc uc uc | uc uc uc uc | uc uc uc uc |
| NADH dehydrogenase iron-sulfur protein 8 | uc uc uc uc | uc uc uc uc | uc uc uc uc | uc uc uc uc |
| NADH dehydrogenase 1 α subcomplex subunit 4 | uc uc uc uc | uc uc uc uc | uc uc uc uc | uc uc uc uc |
| NADH dehydrogenase 1 α subcomplex subunit 5 | ++ uc uc uc uc | uc uc uc uc | uc uc uc uc | uc uc uc uc |
| NADH dehydrogenase 1 α subcomplex subunit 9 | ++ uc uc uc uc | uc uc uc uc | uc uc uc uc | uc uc uc uc |
| NADH dehydrogenase 1 α subcomplex subunit 10 | ++ uc uc uc uc | uc uc uc uc | uc uc uc uc | uc uc uc uc |
| NADH-ubiquinone oxidoreductase chain 1 | ++ uc uc uc uc | uc uc uc uc | uc uc uc uc | uc uc uc uc |
| NADH-ubiquinone oxidoreductase chain 4 | ++ uc uc uc uc | uc uc uc uc | uc uc uc uc | uc uc uc uc |
| NADH-ubiquinone oxidoreductase chain 5 | ++ uc uc uc uc | uc uc uc uc | uc uc uc uc | uc uc uc uc |
| NADH dehydrogenase flavoprotein 2      | ++ uc uc uc uc | uc uc uc uc | uc uc uc uc | uc uc uc uc |
| Complex II                              |           |           |           |           |
| Succinate dehydrogenase flavoprotein subunit | +++       | +         | uc uc uc uc | uc uc uc uc |
| Complex III                             |           |           |           |           |
| Cytochrome b-c1 complex subunit 1      | –         | –         | –         | –         |
| Cytochrome b-c1 complex subunit 2      | +         | ++        | ++ uc uc uc | uc uc uc uc |
| Cytochrome b-c1 complex subunit 7      | uc uc uc uc | ++ uc uc uc | uc uc uc uc | uc uc uc uc |
| Complex IV                              |           |           |           |           |
| Cytochrome c oxidase subunit 2         | –         | –         | ++ uc uc uc | uc uc uc uc |
| Cytochrome c oxidase subunit 4 isoform 1 | ++ uc uc uc | ++ uc uc uc | uc uc uc uc | uc uc uc uc |
| Cytochrome c oxidase subunit 5A        | ++ uc uc uc | ++ uc uc uc | ++ uc uc uc | uc uc uc uc |
| Cytochrome c oxidase subunit 5B        | uc uc uc uc | ++ uc uc uc | ++ uc uc uc | uc uc uc uc |
| Cytochrome c oxidase subunit 6C        | ++ uc uc uc | ++ uc uc uc | ++ uc uc uc | uc uc uc uc |
| Cytochrome c oxidase subunit 7A2       | ++ uc uc uc | ++ uc uc uc | ++ uc uc uc | uc uc uc uc |
| HIG1 domain family member 1A           | ++ uc uc uc | ++ uc uc uc | ++ uc uc uc | uc uc uc uc |
| Complex V                               |           |           |           |           |
| α                                       | uc uc uc uc | uc uc uc uc | uc uc uc uc | uc uc uc uc |
| β                                       | ++ uc uc uc | ++ uc uc uc | uc uc uc uc | uc uc uc uc |
| γ                                       | ++ uc uc uc | ++ uc uc uc | uc uc uc uc | uc uc uc uc |
| a                                       | ++ uc uc uc | ++ uc uc uc | uc uc uc uc | uc uc uc uc |
| b                                       | ++ uc uc uc | ++ uc uc uc | uc uc uc uc | uc uc uc uc |
| d                                       | ++ uc uc uc | ++ uc uc uc | uc uc uc uc | uc uc uc uc |
| f                                       | ++ uc uc uc | ++ uc uc uc | uc uc uc uc | uc uc uc uc |
| g                                       | ++ uc uc uc | ++ uc uc uc | uc uc uc uc | uc uc uc uc |
| O                                       | uc uc uc uc | ++ uc uc uc | uc uc uc uc | uc uc uc uc |
| F1 complex assembly factor 1            | ++ uc uc uc | ++ uc uc uc | uc uc uc uc | uc uc uc uc |
| Glycolysis                              |           |           |           |           |
| Hexokinase                              | ++ uc uc uc | ++ uc uc uc | ++ uc uc uc | ++ uc uc uc |
| Glucokinase                             | ++ uc uc uc | ++ uc uc uc | ++ uc uc uc | ++ uc uc uc |
| Phosphoglucone isomerase                | ++ uc uc uc | ++ uc uc uc | ++ uc uc uc | ++ uc uc uc |
| Phosphofructokinase                     | ++ uc uc uc | ++ uc uc uc | ++ uc uc uc | ++ uc uc uc |

(continued on next page)
function increased, which suggested an intense phase of protein translation and cellular reprogramming. In particular, enzymes that were involved in glycolysis/gluconeogenesis (>20 proteins), oxphos (>15 proteins), branched-chain amino acid degradation (>10 proteins), and the TCA cycle (>10 proteins) increased their content by a factor above +20% (P < 0.05).

Sequential construction of the oxphos system during human podocyte differentiation

Components of oxphos did not increase simultaneously during the 3 phases of differentiation introduced above (Table 2). Subunits of respiratory chain complex IV and V were increased at later stages compared with complex I. In particular, synthesis of the α- and β-subunits of the F1 catalytic-domain of the F1F0 ATP synthase complex was increased at the final stages of podocyte differentiation (Table 2 and Fig. 2E). Proteomic analysis also revealed that most glycolytic enzymes were decreased during podocyte differentiation (Table 2). In accordance with an oxidative shift, proteins that were involved in the fueling of oxphos with fatty acid, amino acids, or ketones were also up-regulated (Table 2).

A glycolytic switch occurs in podocytes under high glucose conditions

Premature podocytes that were cultured from d 7 to 15 in media with 8 different glucose concentrations showed an increase in lactate levels (Fig. 3A) that was suggestive of a glycolytic switch. Although hyperglycemia is clinically observed when glucose concentration ranges above 7 mM, diabetes symptoms do not typically become noticeable until 15–20 mM. To mimic this situation, we conducted in vitro studies with 20 mM glucose. Podocyte dependency on oxphos for cellular ATP production was decreased in high glucose (HG; 20 mM), whereas that of glycolysis increased compared with podocytes cultured in normal glucose (5 mM; Fig. 3B). This metabolic switch was explained, in part, by a reduction of mitochondrial biogenesis, as PGC-1α, NRF-1, and TFAM expression and DNA content were reduced in HG conditions (Fig. 3C). Accordingly, activity of citrate synthase, an indicator of mitochondrial active content, was also decreased in those conditions (Fig. 3C).

### Table 2. (continued)

| Changes of energy metabolism in podocytes | Stage (d) | | | |
|-----------------------------------------|-----------|-----------|-----------|-----------|
| Aldolase                                | 0–3       | 3–7       | 7–15      | HG/NG     |
| Triose phosphate isomerase              | ++        | uc        | uc        | uc        |
| Glyceraldehyde phosphate dehydrogenase  | uc        | uc        | uc        | uc        |
| Phosphoglycerate kinase                 | ++        | uc        | uc        | uc        |
| Phosphoglyceromutase                    | uc        | uc        | uc        | uc        |
| Enolase α                               | uc        | uc        | uc        | uc        |
| Enolase γ                               | uc        | uc        | uc        | uc        |
| Pyruvate kinase                         | +         | +         | uc        | +         |
| Lactate dehydrogenase                   | uc        | uc        | uc        | uc        |
| β-Oxidation                             | uc        | uc        | uc        | uc        |
| Acyl-CoA synthetase                     | uc        | uc        | uc        | uc        |
| Acyl-CoA dehydrogenase (very long chain)| uc        | uc        | uc        | uc        |
| Acyl-CoA dehydrogenase (medium chain)   | uc        | uc        | uc        | uc        |
| Enoyl-CoA hydratase                     | uc        | uc        | uc        | uc        |
| 3-Hydroxyacyl-CoA dehydrogenase         | uc        | uc        | uc        | uc        |
| β-Ketoacyl-CoA thiolase                  | uc        | uc        | uc        | uc        |
| Ketone body degradation                  | uc        | uc        | uc        | uc        |
| Succinyl-CoA transferase                 | uc        | uc        | uc        | uc        |
| Amino acid degradation                   | uc        | uc        | uc        | uc        |
| Serine hydroxymethyltransferase          | uc        | uc        | uc        | uc        |
| Glutamate dehydrogenase 1               | uc        | uc        | uc        | uc        |
| Glutaminolysis                          | uc        | uc        | uc        | uc        |
| Glutamate dehydrogenase 2               | uc        | uc        | uc        | uc        |
| ATP/ADP translocator                    | uc        | uc        | uc        | uc        |

These data were obtained by using quantitative proteomics. Only proteins that showed >20% change in their expression levels with statistical significance (P < 0.05) between 2 groups were selected. NG, normal glucose; uc, no change during the indicated period; ++++, >10× change; +++, 5–10×; +, 2–5×; ++, 1.5–2.0×; +, 1.2–1.5×; −−−, <0.1×; −−−−, 0.1–0.2×; −−−−, 0.2–0.5×; −−−−, 0.5–0.67×; −−−−, 0.67–0.8×.
Figure 3. A) Analyses of culture medium from d 11–13 (left: glucose concentrations 0, 2.5, 5, 7.5, 10, 15, 20, and 30 mM from left to right, respectively; right: culture medium with 5 mM glucose, with 5 mM glucose and 5 mM mannitol, and with 5 mM glucose and 15 mM mannitol from left to right, respectively). Representative images of the culture medium (top). pH of the culture media (middle). Lactate levels in culture media (bottom). *P values by 1-way ANOVA are indicated in graphs. B) Measurement of podocyte ATP content when cultured in normal glucose (NG; 5 mM) or HG (20 mM) condition. Total ATP, mitochondrial ATP, and glycolytic ATP content in podocytes, and contribution of oxphos to the total ATP content were analyzed. C) PGC-1α mRNA (continued on next page)
HG triggers metabolic reprogramming in human podocytes in vitro

We performed a differential proteomic analysis between podocytes that were grown in 20 mM (HG) or those grown in 5 mM glucose (normal glucose) between d 7 13. Thirty-one mitochondrial proteins were decreased in podocytes that were cultured under HG (Table 1). Oxphos was severely impacted, with >10 proteins reduced. Key components of intermediate metabolism, such as glutaminolysis or TCA cycle, were altered (Table 2). On the other hand, at the level of the respiratory chain, key components of complexes III, IV, and V were decreased (Table 2), as well as key components of ADP phosphorylation, such as ATP synthase (Fig. 2E), ANT2, and the mitochondrial phosphate carrier. Measurement of respiratory chain enzyme activities confirmed a reduction for complexes III and IV (Fig. 3D). On the other hand, the content of 5 glycolytic enzymes was increased in HG conditions (Table 2). Lactate dehydrogenase A was also increased in HG (+48%), which was consistent with the elevated lactate production described in Fig. 3A. Lastly, proteomic analysis showed that antioxidant enzymes, such as catalase (+86%), glutathione S transferase (+65%), glutathione S transferase Ω1 (+21%), thioredoxin reductase (+59%), and peroxiredoxin 2 (+51%) were increased in hyperglycemia. This could be consecutive to the higher steady-state of H2O2 levels that were measured in the HG conditions (Fig. 3E).

Regulation of HG-mediated podocyte glycolytic switch

First, we observed an alteration of the LKB1-AMPK pathway—a master regulator of oxphos—in podocytes that were grown under HG conditions (Fig. 3F). Analysis of the mRNA content of 84 genes that are involved in glucose metabolism (Fig. 4A) also showed that hyperglycemia stimulated glycolysis but also activated expression of the oxphos inhibitor, pyruvate dehydrogenase kinase 1 (PDK1) (25). Search for potential transcription factors involved in metabolic reprogramming induced by hyperglycemia (Fig. 4B) showed a decrease in MEF2C and MYF5. A previous study revealed that MEF2C controls MYF5 expression (Fig. 4D). In these conditions, dual inhibition of MEF2C and MYF5 also altered podocin expression (Fig. 4E). Of note, use of a single esiRNA against MEF2C or MYF5 alone did not change the levels of complex IV, PGC-1α, or podocin (Fig. 4C–E). Such a cooperative effect between MEF2C and MYF5 was previously described for the synergistic transactivation of a myogenin (27). We further observed that MEF2C controlled MYF5 expression (Fig. 4F), whereas the opposite did not occur (data not shown). In addition to energy metabolism and the role of MEF2C-MYF5 cooperation in reducing PGC-1α and oxphos, changes in the cellular signaling pathways of podocytes challenged with HG are listed in Table 3. As discussed above, NRF-2-mediated oxidative stress response was identified by Ingenuity Pathway Analysis software as activated under HG conditions, as well as glycolysis and mitochondrial dysfunction.

Investigation of the glycolytic switch in diabetic nephropathy kidney samples

Because proteomics showed up-regulation of the glycolytic enzyme, pyruvate kinase M2 podocyte, under HG (Table 2), and given the role of this enzyme in anabolic glycolysis (28), we measured PKM2 expression in human kidney sections (Fig. 5). Intense labeling was found in podocytes and Bowman’s capsules in glomeruli, and PKM2-positive podocytes were significantly more numerous in diabetic kidney patients (Fig. 5). Furthermore, expression of MYF5 and MEF2C, which were detected as decreased transcriptomic factors in our in vitro study, was significantly decreased in patients with diabetic nephropathy (Fig. 5). Likewise, intensities of PGC-1α expression were also down-regulated in samples from patients with diabetic nephropathy (Fig. 5).

DISCUSSION

Mitochondria are increasingly recognized as key players in acquired renal diseases (29), and Reidy et al. (30) recently reviewed their role in diabetic kidney disease initiation. In the present study, we described the metabolic reprogramming of human podocytes that are confronted with HG levels and we demonstrated the role of MEF2C and MYF5 in the glycolytic switch. Our work has implications for the fundamental understanding of human podocyte differentiation, but also for the study of diabetic nephropathy, as we could validate some of the in vitro findings on human kidney samples that were obtained from patients with diabetes. Regarding the analysis of the determinants of podocyte differentiation, our study has relevance as podocyte dedifferentiation may occur in the context of podocyte...
Figure 4. A, B) Changes of mRNA level of 84 genes involved in glucose metabolism (A) or DNA transcription (B) between podocytes cultured in normal glucose (NG) or HG as analyzed by quantitative PCR microarray. Tables in panels show genes whose expression levels were statistically different between both groups. A scheme of the main regulatory network identified in podocyte remodeling by hyperglycemia. C–F) Complex IV (C), PGC-1α (D), and podocin (E) expression of podocytes cultured with esiRNA of EGFP, MYF5 (F), MEF2C, and MYF5 + MEF2C were determined by Western blotting.
injuries (17, 18). Moreover, previous analyses of the alteration of mitochondrial bioenergetics were performed in diabetic podocytes that were obtained from rats and mice, which could differ from human podocytes (31, 32) or between different mouse strains (33). Moreover, the situation is unclear in mice podocytes, as Wang et al. (34) reported an alteration of oxphos by hyperglycemia, whereas other authors showed the opposite (10, 35). In contrast with mice or rat podocyte studies, our data were obtained from human podocytes and from kidney sections from patients with diabetic nephropathy.

A novel finding of our study is that MEF2C and MYF5 were involved in the bioenergetic dedifferentiation of human podocytes toward oxphos, and that hyperglycemia altered these transcription factors. In addition, we described a sequential change in the energetic machinery without precedent. We found that glycolytic enzymes are immediately repressed when podocyte differentiation occurs and that respiratory chain proteins increase in a specific order. In particular, the components of the F1F0-ATP synthase complex are only built up when the rest of the machinery has been produced, which could potentially be to avoid hydrolysis of glycolytic ATP by this complex. A recent study showed that the respiratory chain complex III assembly depends on the energy state of mitochondria (36); thus, feedback mechanisms could also control synthesis of ATP synthases and the overall maturation of oxphos. Another feature of podocyte differentiation resides in the stimulation of oxidative fueling processes. Fatty acids and branched-chain amino acids are excellent fuels for oxphos in skeletal muscle and the heart, and our findings suggest that those energy substrates could also play an important role in podocyte physiology (37). Our findings indicate that onset of oxphos that was observed during normal podocyte differentiation (5 mM glucose) required expression of both MEF2C and MYF5, but also that of TFAM, NRF-1, and PGC-1α (38).

The second part of our work focused on the toxic effect of HG levels (in vitro) and of hyperglycemia (in vivo) on podocyte bioenergetic apparatus. Podocytes that were exposed to 20 mM glucose not only aborted the bioenergetic differentiation process discussed above, but they also shifted to a different program that favored glycolysis (bioenergetic dedifferentiation). Alteration in mitochondrial F1F0-ATP synthase subunits might impact mitochondrial structure (39), dynamics, and function (40) in podocytes, thereby leading to foot process effacement as an early pathologic change in diabetic nephropathy (2). Of interest, knockdown of MEF2C and MYF5 also triggered the reduction of podocin expression in our in vitro study, which suggested a more global dedifferentiation process caused by hyperglycemia. Glycolytic switch observed in podocytes that were grown in 20 mM glucose resembles the Warburg effect described in cancer cells wherein a reduction of PGC-1α content and mitochondrial biogenesis was previously reported (41). Increase in oxidative stress triggered by HG growth conditions in our in vitro study also correlates with the consequences of hyperglycemia in

| Name                                              | P        | Overlap or no. of molecules |
|---------------------------------------------------|----------|-----------------------------|
| Glycolysis I                                       | 2.98E-05 | 16.7%                       |
| NRF-2-mediated oxidative stress response          | 1.60E-04 | 3.9%                        |
| Mitochondrial dysfunction                         | 1.78E-03 | 3.6%                        |
| Ahl hydrocarbon receptor signaling                 | 5.11E-03 | 3.4%                        |
| Renal necrosis/cell death                          | 1.12E-02 | 1.9%                        |
| Hereditary disorder                                | 1.51E-02 | 54                          |
| Cellular growth and proliferation                  | 1.43E-02 | 59                          |
| Kidney failure                                     | 4.52E-01 | 4                           |
| Renal inflammation                                 | 1.73E-01 | 4                           |
| Renal nephritis                                    | 1.73E-01 | 4                           |
| Nephritis                                          | 8.72E-02 | 2                           |
| Renal necrosis/cell death                          | 4.91E-01 | 5                           |
| Potential regulators (transcriptional)             |          |                             |
| Estrogen-related receptor α                        | 2.08E-06 |                             |
| cAMP-responsive element binding protein 3-like 1   | 4.67E-05 |                             |
| Functional networks                                |          |                             |
| Cell death and survival, cellular development, cell|          |                             |
| growth and proliferation                           |          |                             |
| Skeletal and muscular disorders, hereditary disorder, development disorder |          |                             |
| Top proteins up-regulated (fold-change)            |          |                             |
| TOP2A (4.011); DDX42 (2.389); IPO5 (1.893); CSNK2A1 (1.746); TGM2 (1.715); IGF2R (1.654); CALU (1.654); PSNB3 (1.636); MYL12A (1.608); RNF215 (1.580) | |                             |
| Top proteins down-regulated                        |          |                             |
| HSPE1 (−2.439); UCHL1 (−2.185); MAN2A1 (−2.141); HADHA (−2.138); CYB5R3 (−2.100); ITGAV (−2.077); ATP5B (−2.073); ABCE1 (−2.060); FLNC (−1.976); LDHB (−1.947) | |                             |

TABLE 3. Summary of the top biochemical pathways and transcriptional regulators involved in energy metabolism impacted by hyperglycemic condition (Ingenuity Pathway Analysis)
humans (42). In different models, mechanisms of HG-mediated reactive oxygen species increase involved the alteration of mitochondrial respiration (43), which suggested the importance of restoring mitochondrial bioenergetics in this disease. In our study, human podocytes that were confronted with HG showed an increase in oxidative stress despite up-regulation of several antioxidant defenses proteins. In patients with diabetes, advanced glycation end products that induced increased oxidative stress were considered therapeutic targets for using Kremezin and benfotiamine, 2 U.S. Food and Drug Administration–approved advanced glycation end product inhibitors (44).

Regarding the signaling mechanisms that are involved in hyperglycemia-induced metabolic shift in podocytes, we observed that HG and hyperglycemia reduced the expression of MEF2C, which participates in control of the expression of numerous components of oxidative metabolism, notably PGC-1α (41). Previous findings demonstrated the association between MEF2C and the AMPK axis during exercise training in skeletal muscle (45), and our findings revealed that HG altered both the activation of AMPK and the protein content of AMPK activator LKB1 (24, 46, 47). Of interest, we observed that only a combined inhibition of MEF2C and MYF5 expression with esiRNA was required to block the oxphos shift. This could suggest that both transcription factors cooperate at the site of gene transcription to induce expression of target genes. In agreement with our findings, a synergistic transactivation of a myogenin by Myf5 and MEF2 was previously demonstrated (27). The recently identified RNA-binding activity of MYF5 (48) might also suggest the need to conduct further genetic investigations on the collaboration between MEF2C and MYF5. In addition, we observed

Figure 5. Representative images of immunostaining of kidney-biopsied specimens from normal participants and patients with diabetic nephropathy. Arrows indicate positive podocytes. Percentages of positive cells for PGC-1α, MYF5, and MEF2 per total cells in glomeruli were calculated. For pyruvate kinase, numbers of positive podocytes per glomerulus were counted. *P < 0.05, **P < 0.01, unpaired Student’s t test.
up-regulation of PDK1, a critical component of the Warburg effect that is capable of shutting down glucose-dependent oxphos in situations of pseudohypoxia (49) or in conditions of high fatty acid and HG intake in pancreatic β-cells (50). The link between MEF2C and PDK1 remains to be established. Lastly, our signaling study showed that mTOR expression was inhibited in podocytes that were cultured in HG medium, as expected in conditions of LKB1-AMPK axis activation. This is consistent with previous studies that have shown that mTOR is activated in podocytes in diabetic nephropathy (51, 52). Recently, mTOR inactivation (52, 53), restoration of the LKB1-AMPK activity (54), and PGC-1α activation (55) were proposed as novel potential therapeutic options for diabetic nephropathy. Our findings further suggest that stimulation of MEF2C and MYF5 expression could permit the restoration of podocyte bioenergetics and mitochondrial biogenesis in these conditions.

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AUTHOR CONTRIBUTIONS

Tos. Imasawa performed most of the experiments, designed the study, and wrote the manuscript; E. Obre, N. Bellance, J. Lavie, Tom. Imasawa, and G. Benard performed cell culture, cell staining, quantitative PCR, Western blotting, and quantitative PCR microarray, and analyzed the data; C. Rigothier, Y. Delmas, C. Combe, and D. Lacombe performed and organized the generation of cell culture system and designed a part of this study; S. Claverol and M. Bonneu performed all proteomics studies; and R. Rossignol performed most of the bioinformatics studies and largely participated in constructing study design and methods as well as writing the manuscript.

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