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Differential impact of glucose levels and advanced glycation end-products on tubular cell viability and pro-inflammatory/profibrotic functions

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ABSTRACT

High glucose (HG) or synthetic advanced glycation end-products (AGE) conditions are generally used to mimic diabetes in cellular models. Both models have shown an increase of apoptosis, oxidative stress and pro-inflammatory cytokine production in tubular cells. However, the impact of the two conditions combined has rarely been studied. In addition, the impact of glucose level variation due to cellular consumption is not clearly characterized in such experiments. Therefore, the aim of this study was to compare the effect of HG and AGE separately and of both on tubular cell phenotype changes in the HK2 cell line. Moreover, glucose consumption was monitored every hour to maintain the glucose level by supplementation throughout the experiments. We thus observed a significant decrease of apoptosis and H_2O_2 production in the HK2 cell. HG or AGE treatment induced an increase of total and mitochondrial apoptosis as well as TGF-β release compared to control conditions; however, AGE or HG led to apoptosis preferentially involving the mitochondria pathway. No cumulative effect of HG and AGE treatment was observed on apoptosis. However, a pretreatment with RAGE antibodies partially abolished the apoptotic effect of HG and completely abolished the apoptotic effect of AGE. In conclusion, tubular cells are sensitive to the lack of glucose as well as to the HG and AGE treatments, the AGE effect being more deleterious than the HG effect. Absence of a potential synergistic effect of HG and AGE could indicate that they act through a common pathway, possibly via the activation of the RAGE receptors.

1. Introduction

Diabetic nephropathy (DN) remains the main cause of end-stage renal disease and is still a challenge [1], affecting vascular, glomerular and tubular components. Several pathways are studied in glomerular cells involving polyol and hexosamine pathways [2], such as oxidative stress [3]. Nonenzymatic linkage of glucose with amino groups leads to advanced glycation end-products (AGE), whose levels increase in diabetes [4] and chronic kidney diseases [5]. They are responsible for oxidative stress by inducing reactive oxygen species (ROS) production. ROS production is triggered by PKC, NADPH oxydase (NOX) or mitochondrial activation [6]. Furthermore, AGE and HG induce *TGF-β1* activation and ROS, leading to increased connective tissue growth factor [7], followed by extracellular matrix expansion, epithelial–mesenchymal transition and tubular atrophy [8]. Tubular injuries in DN have only recently been investigated and seem to be preferentially associated with the loss of kidney function rather than glomerular lesions [9].

Two *in vitro* conditions are currently available to mimic diabetes: high glucose (HG) and AGE exposure. However, only few studies have analyzed the effect of HG and AGE together. The effects of

Abbreviations: AGE, advanced glycation end-products; AGE-BSA, glycated bovine serum albumin; BSA, bovine serum albumin; DHR, Dihydrorhodamine; DIOC6, 3,3′-dihexyloxacarbocyanine iodide; DMEM, Dulbecco's minimum essential medium; DN, diabetic nephropathy; GLUT, glucose transporter; HG, high glucose condition; KSFM, keratinocyte-serum free medium; LG, low-glucose condition; M, Mannitol condition; NOX, NADPH oxidase; RAGE, receptor for advanced glycation end-product; ROS, reactive oxygen species; SGLT2, sodium/glucose co-transporter 2; TGF β, transforming growth factor beta.

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AGE involve its extracellular accumulation [10] and intracellular pathway activation mainly through the activation of the receptor for advanced glycation end-products (RAGE) [11]. An AGE increase may be driven by an excess of its intake or an increase of its synthesis. In addition, a high glucose level in culture medium leads to extracellular synthesis of AGE through the Maillard reaction as well as the intracellular production of methylglyoxal. Thus the HG condition may lead to changes in the tubular cell phenotype by a proper effect or by a local AGE synthesis effect and RAGE axis stimulation. On the other hand, the glucose level decrease during cell culture may induce a "hypoglycemic side-effect" [12], hypoglycemia being clinically defined as a blood glucose level below 2.8 mmol/L [13]. Therefore, this study was conducted (1) to quantify the glucose decrease during HK-2 cell culture and to analyze its impact on cellular survival in order to control the glucose level by supplementation and (2) to compare the HG, AGE and HG + AGE impacts on apoptosis, oxidative stress and the inflammatory response of HK2 cells in culture.

2. Material and methods

2.1. Cell culture and reagents

HK-2 cells, a proximal tubular cell line immortalized by HPV-16, were obtained from American Type Cell Collection (Molsheim, France), expended in keratinocyte-SF medium supplemented with L-glutamine, 5 ng/mL epidermal growth factor and 50 μg/mL bovine pituitary extract (from Invitrogen; Saint Aubin, France), at 37 °C, 5% CO_2. At 80% confluence, cells were growth-arrested for 24 h in serum-free Dulbecco's MEM (DMEM) from Sigma–Aldrich (St-Quentin-Falavier, France). Growth-arrested cells were exposed to the following conditions up to 72 h: KSFM as a control experiment or DMEM that contains 5.6 mmol/L glucose or without further supplementation for low-glucose experiments (LG) or with 25 mmol/L D-mannitol (M) or 25 mmol/L D-glucose for high-glucose conditions (HG) or 100 mg/L bovine serum albumin (BSA) from Sigma–Aldrich, 100 mg/L AGE-BSA (AGE) or 25 mmol/L glucose and 100 mg/L AGE-BSA (AGE+).

2.2. Advanced glycation end-product (AGE) synthesis

As previously described [14], AGEs were produced by incubating 20 mg/mL of BSA with 0.1 M D-glyceraldehyde in 0.2 M phosphate buffer for 12 days. Validation of BSA transformation was assessed by fluorimetry following the appearance of AGE (λ_{ex} 340 nm, λ_{em} 440 nm) and the loss of BSA (λ_{ex} 280 nm, λ_{em} 340 nm) (Varian Cary Eclipse Agilent Technologies, les Ullis, France).

2.3. Glucose supplementation

Glucose concentration was monitored in 50 μL of medium samples. For all analyses, except in experiments without supplementation, 280 mmol/L glucose in DMEM were added every 12 h to obtain a final glucose concentration of 6 mmol/L.

2.4. Apoptosis assay by flow cytometry

Annexin assay (total apoptosis): After trypsin treatment, 10^5 cells were stained with FITC-labeled annexin V and propidium iodide (PI) according to the supplier's recommendations (FITC-annexin V apoptosis detection kit from BD Pharmingen, Rungis, France). Cells positive for annexin V and negative for PI were considered as apoptotic cells.

DIOC6 assay: After trypsin treatment, 10^5 cells were incubated in DMEM with 0.1 μM 3,3′-dihexyloxacarbocyanine iodide (DIOC6) (Sigma–Aldrich) at 37 °C for 30 min [15]. Cells with a loss of fluorescence were counted as apoptotic cells. For each assay, data were collected by flow cytometry (FACS Canto II flow cytometer (Becton Dickinson, San Jose, CA, USA) from 10,000 events and analyzed using Diva® software (BD Biosciences, San Jose, CA, USA).

2.5. Measurement of the pro-apoptotic factor TGF-β1

After exposure of HK-2 cells to the different conditions, culture medium was collected without cell detachment and centrifuged. The supernatant was collected and TGF-β1 concentrations in supernatants were assessed using a colorimetric ELISA kit according to the supplier's recommendations (Quantikine Human *TGF-β1* immunoassay Lille, France). RAGE inhibition was performed with 5 μg/mL RAGE antibody (R&D System, Lille, France), incubated for 2 h before exposing the cells to the above-described different conditions.

2.6. Measurement of H_2O_2 production

Total H_2O_2 production by HK2 cells was measured using the fluorescent probe dihydrorhodamine (DHR, Anaspec, Fremont, France) as described previously [16]. For each assay, data were collected using flow cytometry from 10,000 events and analyzed using Diva® software (same material as for apoptotic assay).

2.7. Statistical analysis

Experiments were evaluated using the Wilcoxon test according to the replicates analyzed and are presented as the median (interquartile). Analyses were performed with *r* software. For all statistical analyses, a p-value < 0.05 was considered significant.

3. Results

3.1. Glucose consumption monitoring during HK-2 cells cultured in LG or HG conditions

The glucose concentration was monitored hourly in the culture supernatant. The glucose concentration decreased linearly in LG (Fig. 1). The same curve slopes were observed between conditions 0.25 mmol/L h^{-1} for LG (r^2 0.995) versus 0.26 mmol/L h^{-1} for HG (R^2 = 0.882), 0.28 mmol/L h^{-1} (r^2 = 0.899) for AGE, and 0.29 mmol/L h^{-1} (r^2 = 0.828) for BSA. However, under the HG condition the decrease was no longer linear but oscillated. The hypoglycemic threshold for plasmatic measurement in humans is defined by a glucose concentration in plasma lower than 2.8 mmol/L. With an initial glucose concentration of 5.6 mmol/L in the culture medium, HK-2 cells reached this "hypoglycemic" threshold after 12 h of culture and no glucose could be detected after 24 h.

3.2. Effect of glucose supplementation on apoptosis and ROS production in HK-2 cells cultured in LG or HG conditions

We analyzed apoptosis and H_2O_2 production of HK-2 cells with or without 12-h supplementation with glucose in LG or HG conditions (Fig. 2). Significant early and sustained H_2O_2 production was observed in control nonsupplemented conditions (p = 0.1 at 24 h, 0.03 at 48 h and 0.009 at 72 h for LG with supplementation versus without) (Fig. 2A). Glucose supplementation significantly reduced the ROS production observed in LG conditions at 24, 48 and 72 h of exposure. Glucose supplementation also protected HK-2 cells