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ORIGINAL ARTICLE

Activation of retinal Müller cells in response to glucose variability

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Abstract

Purpose In the earliest stages of diabetic retinopathy (DR), a dysfunction of Müller cells, characterized by high levels of glial fibrillary acidic protein (GFAP), and aquaporins (AQP), has been observed. Although chronic hyperglycemia causes the activation of Müller cells, the effect of glycemic fluctuations is yet unknown. The aim of the study was to analyze the impact of glucose variability on rat retinal Müller cells (rMC-1) adapted to either normal (5 mM) or high (25 mM) glucose levels.
Methods rMC-1 were cultured in a medium containing either 5 mM (N cells) or 25 mM of glucose (H cells) and then incubated for 96 h in a medium containing (a) low glucose (either 1–3 or 5 mM), (b) basal glucose (either 5 or 25 mM), (c) high glucose (either 25 or 45 mM), (d) basal and high glucose alternated every 24 h; (e) low- and high glucose alternated every 24 h; (f) basal glucose with episodes of low glucose for 30 min twice a day. Müller cells activation was evaluated by measuring the levels of GFAP, AQP4, and phospho-active extracellular signal-regulated kinase (pERK).
Results Under both basal and high glucose concentrations rMC-1 were viable, but their response to glucose excursions was different. In N cells kept under normal (5 mM) glucose, a significant glial activation was measured not only in response to constant high glucose but also to alternating low/high glucose. In H cells, adapted to 25 mM glucose, a significant response was observed only after exposition to a lower (5 mM) glucose concentration.
Conclusion Our results highlight Müller cells activation in response to glucose variability and a different susceptibility depending on the basal glucose conditions.

Keywords Glucose variability · Müller cells activation · Retinal neurodegeneration

Abbreviations

DR	diabetic retinopathy
GFAP	glial fibrillary acidic protein
AQP	aquaporin
rMC-1	retinal Müller cells
pERK	phospho-active extracellular signal-regulated kinase
RGC	retinal ganglion cells
N cells	5 mM (1 g/L) glucose
H cells	25 mM (4.5 g/L) glucose
GLUT1	glucose transporter-1
GLP-1RA	glucagon-like peptide 1 receptor agonists

Introduction

During the last 20 years, diabetic retinopathy (DR) has become one of the most important complications of diabetes and the leading cause of vision impairment and blindness in adults [1]. In this contest, retinal neurodegeneration has recently been considered as an early marker of DR, since it seems to precede vascular damage [2]. The alterations produced by hyperglycemia on the entire neurosensory retina are still poorly understood, and the activation of different metabolic pathways has been proposed.

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In the inner part of the retina, the retinal ganglion cells (RGC) and the retinal nerve fibers represent the neuronal components, early undergoing to diabetes-related apoptosis [3]. As a consequence of RGC dysfunction, the thickness of the retinal nerve fiber layer decreases in diabetic patients with or without mild signs of DR, as detected by the optical coherence tomography. Although very precocious, neuronal apoptosis seems to be preceded by glial cells dysfunction. Müller cells, the major glial component of the retina, are closely associated with astrocytes, endothelial cells, and neurons and play a part in the regulation of the blood–retinal barrier [4]. An increasing number of studies have shown that diabetes is associated with reactive Müller cell gliosis [5], characterized by hypertrophy, cellular proliferation, and increased levels of GFAP and AQPs, the latter playing a crucial role in the water transport through the retina cells and in the modulation of neuronal excitability [6]. In this contest, GFAP and AQPs are commonly utilized as biomarkers of retinal Müller cells activation in in vitro experiments conventionally carried out on cells adapted to normal glucose (5 mM) conditions [7, 8]. However, the concentration of glucose in serum of diabetic animals is more than three times higher than that of control/non-diabetic animals; ~ 23 and 7 mM have been reported in serum of diabetic and control/non-diabetic rabbits, respectively, and similar differences (17 and 5 mM) have been found in the vitreal humor of diabetic and control/non-diabetic rabbits [9]. Moreover, a blood glucose concentration of 23 mM has been measured in a type I diabetic rat model [10]. Therefore, it is very important to choose an appropriate glucose concentration in the culture medium to perform experiments on retinal Müller cells activation [11]. In this study, we determined the effect of 96-h exposure to different glucose concentrations on rat Müller cells routinely maintained not only in normal glucose but also in high glucose to mimic diabetic conditions.

Materials and methods

Cell culture and cell treatments

The immortalized rat retinal Müller cells (rMC-1) were obtained from Kerafast (Kerafast, Boston, MA, USA) [12], and routinely maintained at 37 °C, 5% CO_2 in the Dulbecco's Modified Eagle Medium (DMEM, Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS, Euroclone, Milan, Italy), 100 U/mL of penicillin, 100 mg/mL streptomycin (Sigma-Aldrich), and containing either 5 mM (1 g/L) glucose (N cells) or 25 mM (4.5 g/L) glucose (H cells).

The effect of glucose variability was evaluated on N and H cells plated at a density of 10,000 cells/cm^2 and cultured in glucose-free DMEM supplemented with different glucose concentrations to achieve (a) low-glucose medium (1–3 mM and 5 mM for N and H cells, respectively), (b) basal-glucose medium (5 mM and 25 mM for N and H cells, respectively), (c) high-glucose medium (25 mM and 45 mM for N and H cells, respectively). Cells were incubated for 96 h in a medium containing (a) low glucose; (b) basal glucose; (c) high glucose; (d) basal and high glucose alternated every 24 h; (e) low- and high glucose alternated every 24 h; (f) basal glucose with episodes of low glucose for 30 min twice a day. Mannitol (basal glucose + 20 mM mannitol) (Sigma-Aldrich) was used to rule out the effect of osmolarity in cells exposed to high glucose. Cell culture media in all groups were changed every 24 h. Cells were collected after 96 h and subjected to analysis.

Western blot analysis

Müller cells activation was evaluated by measuring the levels of GFAP, AQP4, and phosphorylated (active) ERK1/2 (pERK).

Cell pellets were washed in PBS and suspended again in RIPA buffer containing 25 mM Tris–HCl (pH 7.6), 150 mM NaCl, 1% Np-40, 1% sodium deoxycholate, 0.1% SDS, 10 mM NaF, 1 mM Na_3VO_4, protease and phosphatase inhibitors (Sigma-Aldrich). After a 10-min incubation on ice, cells were sonicated and lysates were centrifuged at 13,000*g* for 15 min at 4 °C. Protein concentration was then determined on supernatants using the Lowry colorimetric assay. Proteins (40 µg) were loaded on 12% SDS-polyacrylamide gel and then transferred onto a PVDF membrane (GE Healthcare, Chalfont St. Giles, UK). Anti-GFAP (Cell Signaling, Beverly, MA, USA), anti AQP4 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-pERK (Cell Signaling), anti-ERK (Cell Signaling), and anti-β actin (Sigma-Aldrich) were used as primary antibodies. Anti-rabbit or anti-mouse secondary antibodies (Cell Signaling) were revealed with the ECL LiteAblot Extend (EuroClone). The ImageJ software was used to analyze band intensities.

Cytofluorimetric analysis

Cell pellets were fixed with 70% ethanol overnight, stained with propidium iodide (PI) staining solution (3.4 mM Na-citrate, 50 µg/ml PI, 10 µg/ml RNAsi, and 1% Triton X-100) and analyzed by a FACSCalibur (BD Bioscence, San Jose, CA, USA). Flow-cytometric data were analyzed by the FlowJo 8.8.6 software (Tree Stare, Inc, Ashland, OR, USA).

Statistical analysis

Statistical evaluation was done using the Student's *t* test (two tailed). A value of $P < 0.05$ was considered significant for all statistical analyses, which were calculated using GraphPad Prism (GraphPad Software, La Jolla, CA). All the experiments were repeated at least three times, and values are expressed as mean ± SEM.

Results

Basal levels of GFAP, AQP4, and pERK1/2

A preliminary characterization of the rMC-1 cells reveals that the increase of glucose from 5 mM to 25 mM induced an increase in the basal levels of GFAP and phospho-active ERK1/2, while only marginally altered the level of AQP4 (Fig. 1).

Effect of different glucose conditions on GFAP expression

GFAP significantly increased when cells grown in 5 mM glucose (N cells) were exposed for 96 h to both intermittent (5 mM/25 mM) and constant high (25 mM) glucose (Fig. 2a). On the other hand, the level of GFAP gradually decreased when these cells were subjected twice daily to episodes of 30-min low glucose (3 or 1 mM),

A different trend was observed in cells adapted to 25 mM glucose (H cells). Except for a small increase of GFAP in H cells maintained for 96 h in 5 mM glucose, we did not observe significant alterations of GFAP levels in any of the experimental conditions examined (Fig. 2b).

Effect of different glucose conditions on AQP4 expression

AQP4 shows a trend similar to that of GFAP; in rMC-1 adapted to 5 mM glucose, AQP4 is overall higher in cells exposed for 96 h to intermittent or continuous high glucose (25 mM) (Fig. 3a).

On the other hand, in cells adapted to 25 mM glucose, a significant increase of AQP4 is observed only after 96 h of incubation in 5 mM glucose. Of note, the amount of AQP4 decreased when these cells were exposed to 5 mM glucose for 30-min, twice daily (Fig. 3b).

Effect of different glucose conditions on ERK1/2 activation

The trend of ERK1/2 activation in N cells maintained in 5 mM normal glucose conditions is quite similar to that of H cells adapted to 25 mM glucose concentrations. However, the magnitude of change is smaller in H cells. In rMC-1 maintained in 5 mM glucose, a strong increase of pERK was detected when cells were exposed for 96 h to both intermitted and constant high glucose conditions, compared with control cells maintained in basal glucose conditions (Fig. 4a). No activation of ERK1/2 was observed in cells subjected twice daily to episodes of 30 min of low glucose (3 or 1 mM) (Fig. 4a).

In rMC-1 grown in 25 mM glucose, the level of ERK1/2 activation significantly increased after 96-h exposition to constant high glucose (45 mM), while decreased after 96-h exposition to constant 5 mM glucose, compared with control cells maintained in 25 mM glucose (Fig. 4b).

Effect of different glucose conditions on cell cycle

Cytofluorimetric analysis revealed that both types of rMC-1 cells were viable. However, an increase of the hypodiploid sub-G1 phase, suggestive of apoptosis, was observed when cells, adapted to 25 mM glucose, were cultured for 96 h in medium containing 5 mM glucose (Fig. 5b).

Discussion

In vitro studies report that glucose oscillations, like those experienced daily by diabetic patients, are dangerous for some types of cells. This phenomenon is likely a consequence of a higher generation of free radicals during glucose excursions [13–17].

Our study not only showed that prolonged exposure to high glucose is able to induce Müller cells activation, as previous studies already show [18, 19], but for the first time it proved that Müller cells activation occurs also in response to glucose excursions. However, this activation was observed only in cells adapted to normal glucose conditions (5 mM glucose, N cells). Conversely, the chronic exposure of rMC-1 to high glucose (25 mM, H cells) dampens the response to glucose changes. In this case, only a lower glucose concentration (5 mM) is able to induce a further increase of the activation markers GFAP and AQP4. The latter is a water channel protein that is involved in many physiological processes, such as waste removal and maintenance of potassium homeostasis [20]. Moreover, AQP4 has been reported to increase and exert a cytoprotective role in the retina of diabetic rats [8]. In analogy, the increase of AQP4 in our experimental conditions, can be interpreted as a compensatory response to the detrimental effects of high glucose in N cells or of low glucose in H cells, chronically stimulated by high glucose. Overall, these results confirm our recent findings showing that, in type 1 diabetic patients without DR or very mild non-proliferative DR, glucose excursions were positively associated with inner nuclear layer thickness, which includes mainly the nuclei of Müller cells. Therefore, the increase of glycemic variation seems to trigger an early activation of Müller cells [21]. Of note, no

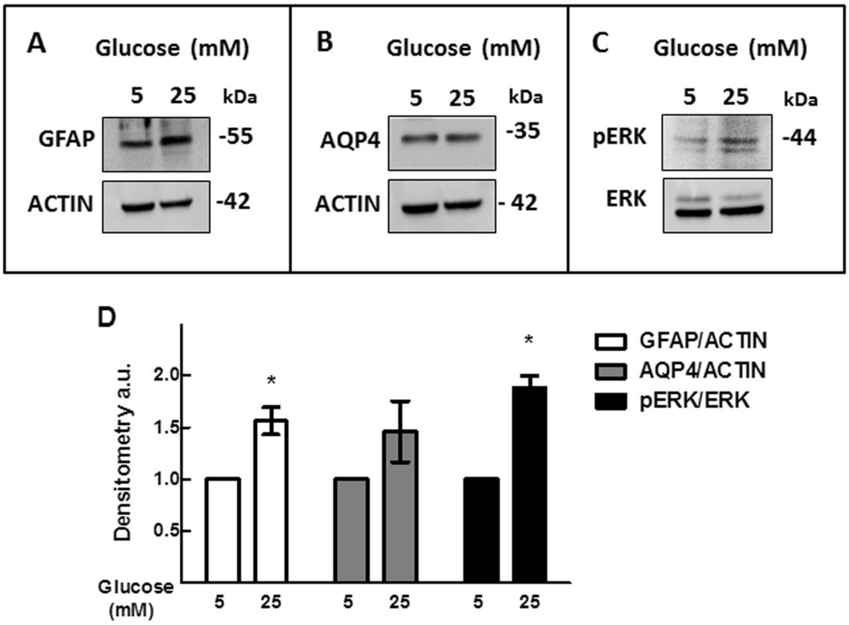

Fig. 1 Basal levels of GFAP, AQP4, and pERK. rMC-1 cells, routinely maintained in the DMEM containing either 5 mM or 25 mM glucose, were lysed and subjected to western blot analysis. **a**, **b** Basal levels of GFAP and AQP4. Beta-actin was used as the loading control. **c** Basal levels of pERK and ERK. **d** Average densitometric quantification of immunoreactive bands. The protein/actin and pERK/ERK ratios in H cells was compared with the ones of the N cells. The results are presented as means ± SEM; $*P < 0.05$ vs N cells maintained in 5 mM glucose

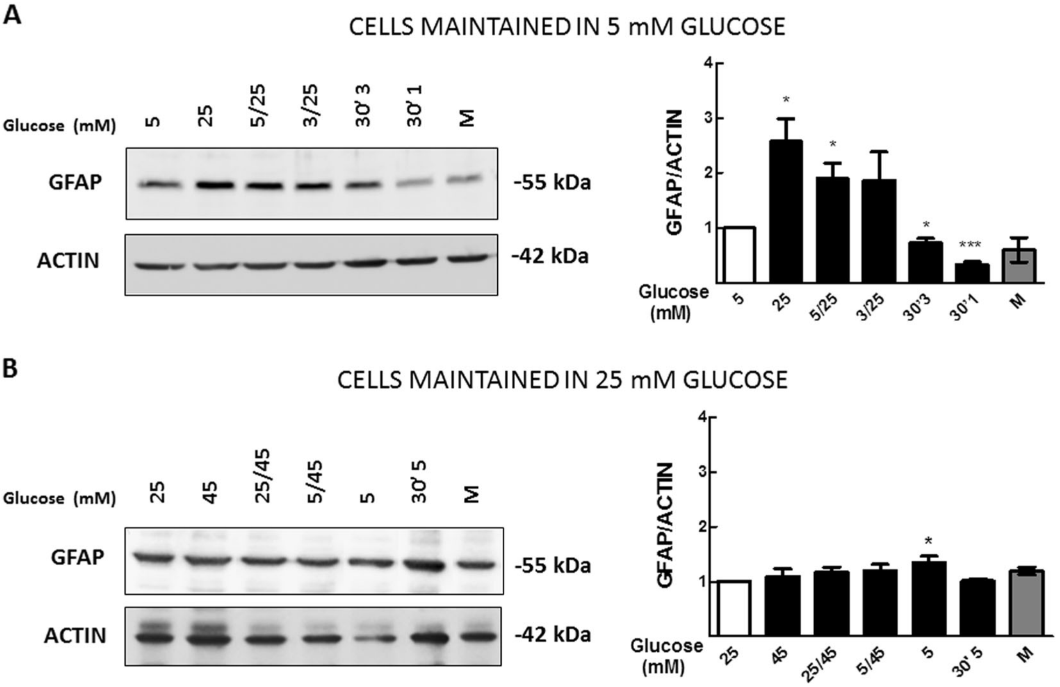

Fig. 2 Effect of glucose fluctuations on GFAP expression. rMC-1 cells, routinely maintained in the DMEM containing (**a**) 5 mM glucose or (**b**) 25 mM glucose, were incubated for 96 h in a medium containing: (5, 25, 45) constant 5 mM, 25 mM, or 45 mM glucose; (3/25) 3 mM or 25 mM glucose alternated every 24 h; (5/25) 5 mM or 25 mM glucose alternated every 24 h; (5/45) 5 mM or 45 mM glucose alternated every 24 h; (25/45) 25 mM or 45 mM glucose alternated every 24 h; (30' 1, 30' 3, 30' 5) basal glucose with episodes of low glucose (1 mM, 3 mM, or 5 mM) for 30 min twice a day; (M) basal level of glucose + 20 mM mannitol. After this time, cells were subjected to western blot analysis to evaluate the amount of GFAP. Left panels: representative immunoblots showing changes in GFAP levels. Right panels: average densitometric quantification of immunoreactive bands of GFAP protein expression under different conditions. Beta-actin was used as the loading control. The GFAP/actin ratio in each group was compared with the one of the cells maintained in the DMEM containing basal levels of glucose. The results are presented as means ± SEM; panel **a**: $*P < 0.05$ and $***P < 0.005$ vs N cells maintained in 5 mM glucose; panel **b**: $*P < 0.05$ vs H cells maintained in 25 mM glucose

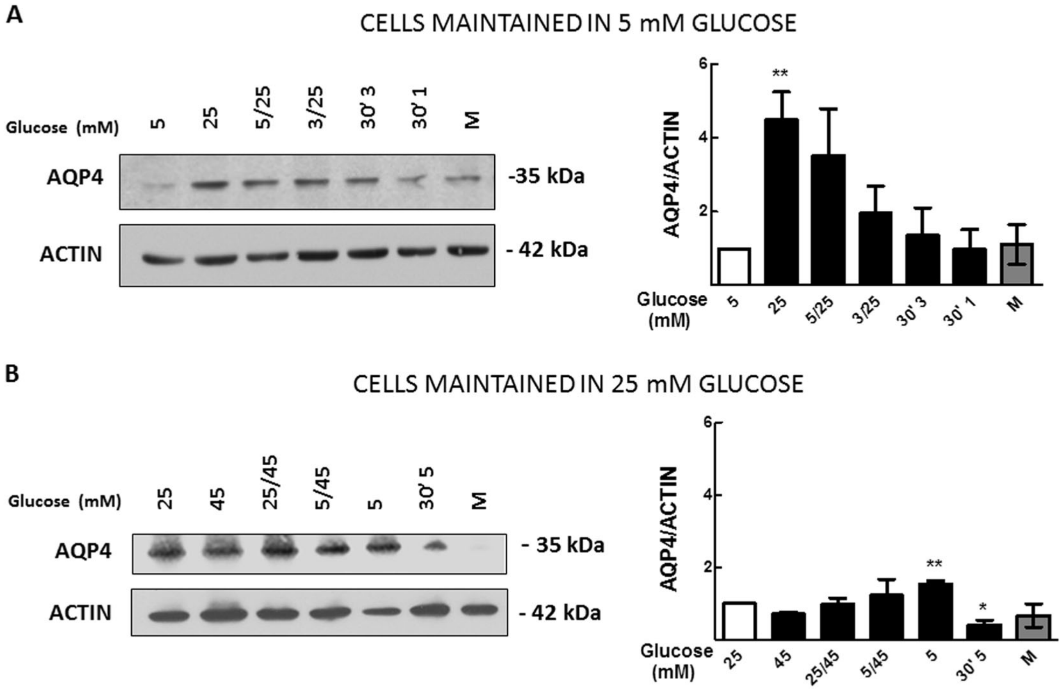

Fig. 3 Effect of glucose fluctuations on AQP4 expression. rMC-1 cells, routinely maintained in the DMEM containing (**a**) 5 mM glucose or (**b**) 25 mM glucose, were cultured for 96 h as reported in Fig. 2. After this time, cells were subjected to western blot analysis to evaluate the amount of AQP4. Left panels: representative immunoblots showing changes in AQP4 levels. Right panels: average densitometric quantification of immunoreactive bands of AQP4 protein expression under different conditions. Beta-actin was used as the loading control. The AQP4/actin ratio in each group was compared with the one of the cells maintained in the DMEM containing basal levels of glucose. The results are presented as means ± SEM; panel **a**: $^{**}P < 0.01$ vs N cells maintained in 5 mM glucose; panel **b**: $^{*}P < 0.05$ and $^{**}P < 0.01$ vs H cells maintained in 25 mM glucose

activation was observed in Müller cells exposed to episodes of low glucose for 30 min (5 mM for H cells, 3 or 1mM for N cells) twice a day. This is possible due to the short time of exposure which was not able to trigger stress responses. It has been reported that at the beginning, reactive Müller cells might indeed have a neuroprotective activity against high glucose-induced neurotoxicity, but then may stop supporting the neurons and contribute to neuronal degeneration [4]. Therefore, we analyzed the ERK1/2 signaling, a prosurvival pathway involved in the neuroprotection of the retina; the activation of ERK1/2 has been observed in both Müller cells of diabetic rat retina and in vitro Müller cells, stimulated with high glucose [22, 23]. In accordance, rMC-1 cells, both in the N and H models, after 96-h exposition to high glucose, showed a strong increase of pERK1/2, when compared with control cells maintained in basal glucose (Fig. 4). However, only for rMC-1 cells adapted to 5 mM glucose, exposure to glucose excursions caused a significant ERK1/2 activation. On the other hand, in rMC-1 adapted to 25 mM glucose, the prolonged exposition to lower (5 mM) glucose levels caused a decrease of pERK. Under these conditions, we also observed an increase of cell death. The amount of cells involved in this process accounts for ~ 15% of total population, and it is indicative of a cellular stress.

This unexpected finding can be explained by considering that ERK1/2 is an essential mediator of cell survival and proliferation [24]; in particular, it has been demonstrated that ERK1/2 activation is required to upregulate the gene expression of the insulin-independent glucose transporter-1 (GLUT1) [25]. Among the family of glucose transporters, GLUT1 is the predominant form expressed in Müller glia and is critical for maintaining energy metabolism in these cells [26]. Another aspect to be considered is the involvement of ERK1/2 in the activation of autophagy by glucose [27]. High levels of glucose cause reactive oxygen species accumulation which in turn activate proteins, such as AMP-activated protein kinase and ERK1/2. The involvement of ERK1/2 in activation of autophagy has been clearly demonstrated [28, 29]. Autophagy is crucial for maintaining cellular homeostasis removing the intracellular organelles or protein aggregates of oxidative damage [30]. For these reasons, our data may suggest that rMC-1 cells adapted to high glucose concentrations become strongly dependent on the glucose-activated ERK signaling for their survival. From this perspective, the decrease of pERK associated to an increase of rMC-1 death should be interpreted as an inability of Müller cells, chronically stimulated by high (25 mM) glucose, to adapt to "normal" (5 mM) glucose values, perceived as "harmful". These data confirmed what was previously observed in the retina of Zucker diabetic fatty rats that became abnormally sensitive to variations in glucose substrate supply under chronic hyperglycemia. In

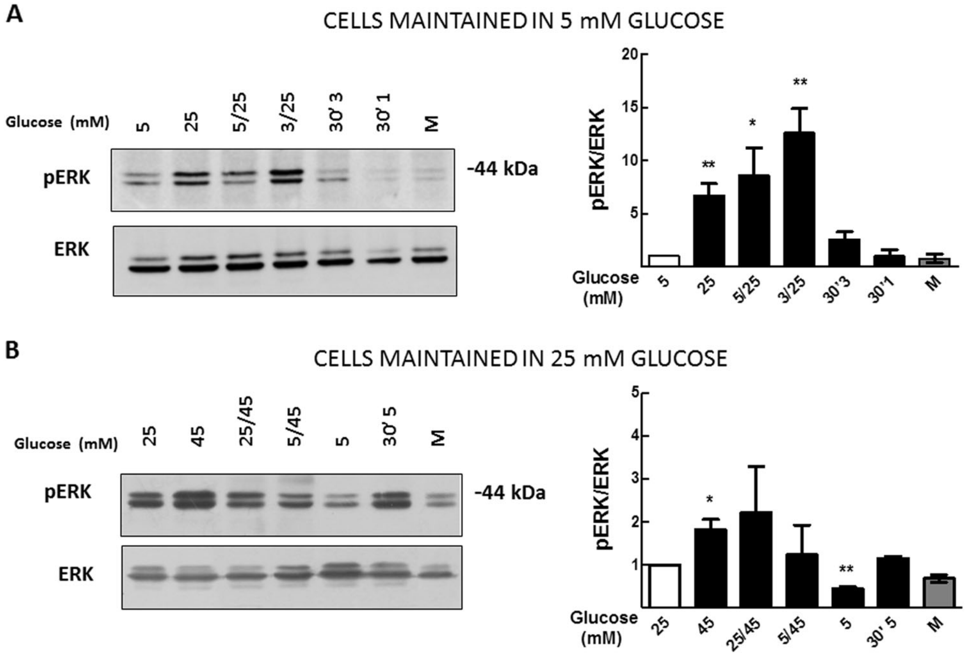

Fig. 4 Effect of Glucose fluctuations on ERK activation. rMC-1 cells, routinely maintained in the DMEM containing (**a**) 5 mM glucose or (**b**) 25 mM glucose, were cultured for 96 h as reported in Fig. 2. After this time, cells were subjected to western blot analysis to evaluate the amount of phosphorylated (active) ERK1/2 (pERK). Left panels: representative immunoblots showing changes in pERK and ERK levels. Right panels: average densitometric quantification of immunoreactive bands of pERK and ERK under different conditions. The pERK/ERK ratio in each group was compared with the one of the cells maintained in the DMEM containing basal levels of glucose. The results are presented as means ± SEM; panel **a**: $*P < 0.05$ and $**P < 0.01$ vs N cells maintained in 5 mM glucose; panel **b**: $*P < 0.05$ and $**P < 0.01$ vs H cells maintained in 25 mM glucose

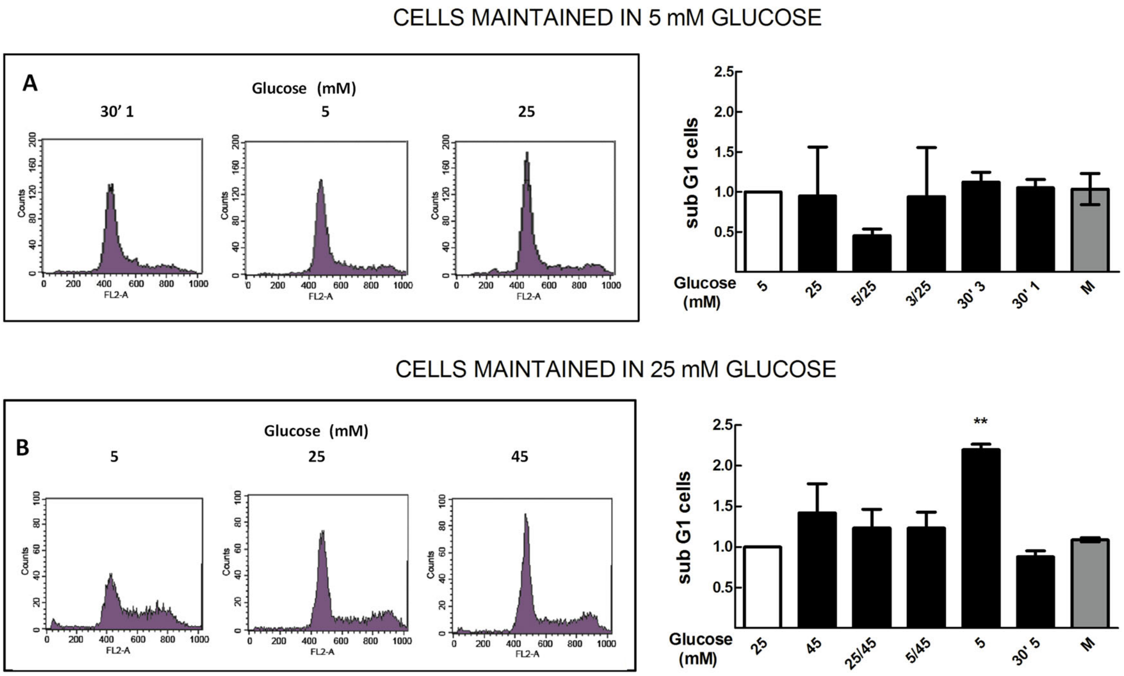

Fig. 5 Flow-cytometry analysis. rMC-1 cells, routinely maintained in the DMEM containing (**a**) 5 mM glucose or (**b**) 25 mM glucose, were cultured for 96 h as reported in Fig. 2. After this time, cells were stained with PI and analyzed to evaluate the percent of population in the various phases of cell cycle. Left panels: representative histograms from three independent experiments. Right panels: average population in the sub-G1 phase under different conditions. The amount of sub-G1 population in each group was compared with the one of the cells maintained in the DMEM containing basal levels of glucose. The results are presented as means ± SEM; $**P < 0.01$ vs H cells maintained in 25 mM glucose

the clinical setting, a similar inability to cope with intensive glucose reduction could make the retina susceptible to damage [31]. Early worsening of DR is a known phenomenon associated with the rapidity and magnitude of improvement in glycaemic control, and it is noteworthy to mention the worsening of DR reported by recent clinical trials with glucagon-like peptide 1 receptor agonists (GLP-1RA). In the SUSTAIN 6 clinical trial programme, the arm treated with semaglutide reported an increased risk of developing DR and the complications of DR, compared with subjects receiving placebo [32]. Varadhan et al. found a progressive worsening of DR in patients treated with exenatide, another drug of the class of GLP-1RA. The authors suggested that the worsening of DR might be explained by the sudden and substantial glycemic compensation caused by treatments, and subsequently found this effect to be transient and reversible [33, 34]. It is therefore tempting to speculate that the dysfunction of Müller cells could play a pivotal role in the early worsening of DR, caused by a rapid achievement of euglycemia.

In conclusion, our data demonstrate a glial activation, in response to glucose excursions already in Müller cells adapted to normal glucose concentrations, confirming our previous results in patients with type 1 diabetes mellitus. Furthermore, we observe a different susceptibility of the Müller cells, depending on the basal glucose conditions, to be considered in the experimental approach for the evaluation of the metabolic signaling involved in neuroretinal damage in the DR.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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