Neuroscience Letters 602 (2015) 56–61

Contents lists available at ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

Long-term exposure to high glucose increases the content of several exocytotic proteins and of vesicular GABA transporter in cultured retinal neural cells

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HIGHLIGHTS

- High glucose increases the content of several exocytotic proteins in retinal cultures.
- Additional exposure to IL-1β did not exacerbate the changes detected in retinal cells.
- Hyperglycemia appears as a major factor contributing for synaptic changes in diabetes.

ARTICLE INFO

Article history:
Received 6 April 2015
Received in revised form 21 June 2015
Accepted 22 June 2015
Available online 30 June 2015

Keywords:
Diabetic retinopathy
Hyperglycemia
Interleukin-1β
Retinal cells
Synaptic proteins
Exocytosis

ABSTRACT

Diabetic retinopathy is a leading cause of vision loss and blindness. Increasing evidence has shown that the neuronal components of the retina are affected even before the detection of vascular lesions. Hyperglycemia is considered the main pathogenic factor for the development of diabetic complications. Nevertheless, other factors like neuroinflammation, might also contribute for neural changes. To clarify whether hyperglycemia can be the main trigger of synaptic changes, we evaluated whether prolonged elevated glucose *per se*, mimicking chronic hyperglycemia, is able to change the content and distribution of several exocytotic proteins and vesicular glutamate and GABA transporters in retinal neurons. Moreover, we also tested the hypothesis that an inflammatory stimulus (interleukin-1β) could exacerbate the effects induced by exposure to elevated glucose, contributing for changes in synaptic proteins in retinal neurons.

Rat retinal neural cells were cultured for 9 days. Cells were exposed to elevated D-glucose (30 mM) or D-mannitol (osmotic control), for 7 days, or were exposed to interleukin-1β (10 ng/ml) or LPS (1 μg/ml) for 24 h. The protein content and distribution of SNARE proteins (SNAP-25, syntaxin-1, VAMP-2), synapsin-1, synaptotagmin-1, rabphilin 3a, VGluT-1 and VGAT, were evaluated by western blotting and immunocytochemistry. The protein content and immunoreactivity of syntaxin-1, synapsin-1, rabphilin 3a and VGAT increased in retinal neural cells exposed to high glucose. No changes were detected when cells were exposed to interleukin-1β, LPS or mannitol *per se*. Particularly, exposure to interleukin-1β for 24 h did not exacerbate the effect of high glucose on the content and immunoreactivity of exocytotic proteins, suggesting the primordial role of hyperglycemia for neuronal changes. In summary, prolonged exposure to elevated glucose alters the total content of several proteins involved in exocytosis, suggesting that hyperglycemia *per se* is a fundamental factor for neuronal changes caused by diabetes.

1. Introduction

Diabetic retinopathy is a leading cause of vision loss and blindness worldwide. Although it has been considered a microvascular disease, increasing evidence has shown that the neural components of the retina are also affected, even before the detection of

Abbreviations: IL-1β, interleukin-1β; LPS, lipopolysaccharide; SNARE, soluble NSF attachment protein; SNAP-25, synaptosome-associated protein with 25 kDa; VAMP-2, vesicle-associated membrane protein 2; VGLUT-1, vesicular glutamate transporter 1; VGAT, vesicular GABA transporter.

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http://dx.doi.org/10.1016/j.neulet.2015.06.044
0304-3940/

microvascular changes [1]. Alterations in electroretinograms in diabetic patients and animals, and loss of color and contrast sensitivity are early signs of neural dysfunction in the retina [2–4]. Moreover, it has been shown that diabetes increases gliosis and apoptosis in neural cells in human and rat retina early in the course of the disease [5–7]. We also showed that long-term exposure (7 days) to elevated glucose increases cell death in primary retinal neural cell cultures [8,9], while others found no signs of neuronal damage in retinal explants after exposure to high glucose for 48 h [10].

Previous studies have suggested that hyperglycemia might not be responsible for all clinical aspects of diabetic retinopathy, since there are numerous cases of this pathology without evident presence of hyperglycemia [11,12], raising the question if hyperglycemia *per se* is necessary or sufficient for the establishment of the disease [1,13]. Despite hyperglycemia being considered the hallmark of this pathology, it also presents characteristics of a low-grade chronic inflammatory disease, characterized by increased production of cytokines, such as interleukin (IL)-1β [14,15].

Previously, we showed that prolonged exposure to high glucose increases [^{3}H]D-aspartate release in retinal neural cell cultures [16], increases the accumulation of extracellular ATP and decreases ATP degradation [17], clearly demonstrating that elevated glucose affects the release of neurotransmitters in retinal cells. Moreover, in high glucose-treated cells, the increase in $[Ca^{2+}]_i$ triggered by KCl, kainate or purinergic P2 receptors activation is enhanced and the recovery to basal Ca^{2+} levels is delayed [18,19], which may account for the increase in neurotransmitter release from retinal neurons. In the retinas of diabetic animals, the release of [^{3}H]D-aspartate evoked by KCl is increased after 4 weeks of diabetes, comparing to the retinas of age-matched control animals [16]. We also found that after 8 weeks of diabetes the evoked GABA release is decreased in retinal synaptosomes, indicating that diabetes might affect neurotransmitter release [20].

Alterations in neurotransmitter release caused by diabetes/hyperglycemia may result from changes in the exocytotic machinery. Previously, we demonstrated that the content of several proteins involved in exocytosis, as well as glutamate and GABA vesicular transporters, and α(1A) subunit of P/Q type calcium channels, can be affected in the retina in nerve terminals [20]. Taking into account these results, and since diabetes is considered a multifactorial disease, we aimed to evaluate whether prolonged elevated glucose *per se* (which mimics chronic hyperglycemic conditions) could affect the content of exocytotic/synaptic proteins and their distribution in retinal neurons. Moreover, since a pro-inflammatory environment develops secondarily in the course of the disease due to hyperglycemia [21], we also aimed to analyze if exposure to an inflammatory stimulus (IL-1β) after prolonged exposure to high glucose could exacerbate the effects induced by elevated glucose in retinal cells, in terms of exocytotic protein content and distribution in retinal neurons. The proteins analyzed control and regulate neurotransmitter release: the SNARE complex proteins (VAMP-2, SNAP-25 and syntaxin-1), synapsin-1, rabphilin 3a, synaptotagmin-1, and vesicular glutamate and GABA transporters. By this work, we aimed to clarify whether hyperglycemia *per se* might be a prevalent feature leading to the molecular changes detected in nerve terminals in the retinas of diabetic animals. Moreover, we aimed to elucidate if inflammation, which develops later as a consequence of hyperglycemia, may aggravate neuronal changes induced by high glucose.

2. Materials and methods

2.1. Primary cultures of rat retinal neural cells

Procedures involving animals were conducted in accordance with EC Directive 86/609/EEC. Retinal cell cultures were obtained from the retinas of 3–5 days-old Wistar rats, as previously described [16]. After 2 days in culture, cells were incubated with 25 mM D-glucose (final concentration 30 mM) or 25 mM D-mannitol (plus 5 mM glucose), which was used as an osmotic control, and maintained for further 7 days in culture. Glucose concentration in control conditions was 5 mM. Cells were also exposed to IL-1β (10 ng/ml) or LPS (1 μg/ml; as a positive control for inflammation) for 24 h in the last day *in vitro*. Cells were used for experimentation on the ninth day of culture.

2.2. Preparation of extracts of cultured retinal cells

Cells were rinsed with ice-cold phosphate-buffered saline (PBS, in mM: 137 NaCl, 2.7 KCl, 10 Na_2HPO_4, 1.8 KH_2PO_4, pH 7.4, at 4 °C) and lysed with RIPA buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.5% DOC, 0.1% SDS, 1 mM DTT) supplemented with complete miniprotease inhibitor cocktail tablets and phosphatase inhibitors (10 mM NaF and 1 mM Na_3VO_4). Lysates were incubated on ice for 30 min and centrifuged at 16,100 × *g* for 10 min at 4 °C. The supernatant was collected and stored at −80 °C until use.

2.3. Western blot analysis

Western blot analysis was performed as previously described [22]. The primary antibodies used are listed in Table 1. Membranes were reprobed and tested for β-actin immunoreactivity (1:5000) to prove that similar amounts of protein were applied in the gels.

2.4. Immunocytochemistry

Retinal cell cultures were washed with PBS and fixed with 4% paraformaldehyde/4% sucrose for 10 min at room temperature (RT). After washing with PBS, cells were permeabilized with 1% Triton X-100 in PBS for 10 min at RT. Non-specific binding was prevented incubating cells with 10% goat serum/0.2% Tween-20 in PBS for 20 min. Cells were then incubated with the primary antibodies (listed in Table 1) for 2 h at RT and then rinsed with PBS and incubated with secondary antibodies Alexa Fluor 594-conjugated anti-mouse IgG (1:250) or Alexa Fluor 488-conjugated anti-rabbit IgG (1:250) for 1 h at RT in the dark. Nuclei were stained with DAPI (1:5,000). Upon rinsing with PBS, the coverslips were mounted on glass slides using Dako mounting medium (Dako, Denmark). Images were acquired in a laser scanning confocal microscope Zeiss LSM 710 (Zeiss, Germany).

2.5. Statistical analysis

Results are presented as mean ± SEM. Statistical significance was determined by using ANOVA, followed by Dunnett's post hoc test. Differences were considered significant for $p < 0.05$.

3. Results

3.1. High glucose increases the content of syntaxin-1 in retinal neural cell cultures

Before evaluating the effects of elevated glucose and IL-1β on the immunoreactivity of exocytotic proteins and vesicular transporter proteins, we analyzed the morphology of retinal neurons by immunocytochemistry using a TUJ-1 (neuron-specific class III beta-tubulin) antibody. Exposure of cultured retinal cells to elevated concentrations of D-glucose (30 mM) or D-mannitol (24.5 mM + 5.5 mM glucose) for 7 days did not induce any alteration in the neuronal morphology (Fig. 1A). Similarly, exposure to IL-1β (10 ng/ml) or LPS (1 μg/ml) for 24 h, or the combinatory exposure