Archives of Biochemistry and Biophysics 647 (2018) 54–66

Contents lists available at ScienceDirect

Archives of Biochemistry and Biophysics

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

Effect of glycated insulin on the blood-brain barrier permeability: An *in vitro* study

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ARTICLE INFO

Keywords:
Type 2 diabetes
Glycated insulin
AGE products
Permeability
Blood-brain barrier

ABSTRACT

Altered blood-brain barrier (BBB) permeability may contribute to pathogenesis of diabetes-related central nervous system disorders. Considering the presence of glycated insulin in plasma of type 2 diabetic patients, we hypothesized that glycated insulin could induce changes in paracellular permeability in BBB. Therefore, the authors decided to study the effect of glycated insulin on paracellular permeability in a BBB model and the change induced in insulin conformation upon glycation.

In this study, the structural modification was examined by fluorescence and circular dichroism spectroscopies and dynamic light scattering. Cell proliferation and production of ROS in astrocytes and HUVEC cells were analyzed by MTT and spectrofluorometric assays, respectively. Apoptosis induction was determined and confirmed by flow cytometry and western blot analyses, respectively. The permeability was measured Lucifer yellow and FITC-Dextran.

According to our results, glycated insulin presented altered conformation and more exposed hydrophobic patches than insulin. Formation of oligomeric species and advanced glycated end products (AGEs) were determined. Lower cell viability, higher apoptosis, and more ROS were detected upon treatment of cells with glycated insulin. Finally, glycated insulin led to increased Lucifer yellow and FITC-dextran transportation across the BBB model which could result from ROS producing and apoptosis-inducing activities of AGE-insulin.

1. Introduction

Type 2 diabetes mellitus (T2DM) is characterized by resistance to insulin, impaired insulin secretion by pancreatic β cells, and high blood glucose levels [1]. Epidemiological studies have shown the relationship between T2DM and the risk of cognitive impairment, dementia, and Alzheimer's disease (AD) [2–6]. Moreover, several studies have shown disturbances of blood brain barrier (BBB) are involved in many central nervous system (CNS) disorders [7]. BBB is an important boundary to protect CNS and peripheral tissues which is comprised of endothelial cells interacting with astrocytic end feet processes [8–10]. Besides, the expression of inter-endothelial tight junctions, transmembrane transport systems, and lack of fenestra aid in developing a highly selective barrier by modulating the integrity of BBB [11,12]. It is believed that astrocytes regulate the expression of tight junction proteins by secreting factors to preserve BBB permeability and integrity [8,13–15].

Insulin, a major regulatory peptide hormone secreted by pancreatic beta cells is involved in controlling metabolism, cognition, and memory in the CNS [16,17]. On one hand, brain microvascular endothelial cells accommodate the transporters engaged in transportation of insulin

Abbreviations: BBB, blood–brain barrier; CNS, central nervous system; AGE, advanced glycated end product; HUVEC, human umbilical vein endothelial cells; T2DM, type 2 diabetes mellitus; AD, Alzheimer's disease; ROS, reactive oxygen species; LY, Lucifer Yellow; FITC-Dextran, Fluorescein isothiocyanate–Dextran; DMEM, Dulbecco's modified Eagle medium; FBS, fetal bovine serum; MTT, (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; Papp, apparent permeability; DMSO, dimethyl sulfoxide; AO/EB, acridine orange/ethidium bromide; SEM, Standard error of mean; ANS, 1-anilinonaphthalene-8-sulfonate; TFA, Trifluoroacetic acid; RP-HPLC, Reversed-phase high performance liquid chromatography; RAGE, Receptor for advanced glycation end products; PBS, Phosphate buffer saline; PET, Polyethylene terephthalate; PVDF, Polyvinylidene difluoride; PMSF, phenylmethylsulphonyl fluoride; IR, insulin receptor; IGF1R, insulin-like growth factor 1 receptor

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https://doi.org/10.1016/j.abb.2018.02.004
Received 2 October 2017; Received in revised form 24 January 2018; Accepted 7 February 2018
Available online 11 February 2018
0003-9861/

from BBB [16]. Furthermore, studies have shown that insulin regulates the integrity and permeability of BBB through increasing endothelial cell proliferation and expression of tight junction proteins [18]. Moreover, insulin has been reported to induce cell proliferation, glycogen storage, and glutamate uptake in astrocytes [19]. Nevertheless, the role of insulin signaling in BBB function has remained obscure [18].

Furthermore, in hyperglycemia conditions like diabetes, glycation process called Maillard reaction occurs as a non-enzymatic and irreversible process in which reducing carbohydrates react with primary amino groups of proteins (amino groups in side chains of arginine and lysine and N-terminal) [20]. Glycation is followed by the emergence of a Schiff base and transformation into Amadori products which rearrange to form advanced glycated end products (AGEs) [21,22]. It has been shown that AGEs could impair structure and function of proteins and induce cell death via increased production of reactive oxygen species (ROS) after interaction with AGE-specific receptor [23]. However, insulin can be modified by glucose and reactive carbonyl compounds through the process of glycation [24–26]. Furthermore, glycated insulin is unable to maintain normoglycemia and lipogenesis in adipose tissue [27,28]. In fact, *in vitro* experiments have shown that Lys 29 in chain B and N-termini of both chains A and B from insulin interact with glucose [26,29].

T2DM and AD are common in insulin resistance and BBB impairment. On the other hand, glycated insulin, which impairs insulin function, has been detected in T2DM patients. Therefore, we hypothesized that induced alteration in insulin conformation mediated by glycation might influence BBB permeability through promoting cytotoxicity in cells comprising BBB. Thus, fluorescence and CD spectroscopic techniques were used to study conformation of insulin upon glycation. In addition, intracellular ROS production, cell viability, and apoptosis in astrocytes and HUVEC cells involved in simulated BBB after treatment with insulin and glycated insulin were determined. Finally, the permeability of BBB was tested by Lucifer Yellow (LY) and Fluorescein isothiocyanate–Dextran (FITC-Dextran) as a tracer probe.

2. Materials and methods

2.1. Materials

Lyophilized human zinc-free insulin, (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), FITC-Dextran (3–5 kDa), Lucifer Yellow, acridine orange, m-aminophenylboronic acid-agarose beads, 1-anilinonaphthalene-8-sulfonate (ANS), fluorescamine, D-glucose, 2′,7′-dichlorofluorescin diacetate, Trifluoroacetic acid (TFA), nonfat-dried milk, hybond-P polyvinylidene difluoride (PVDF) membranes, and polyacrylamide were purchased from Sigma-Aldrich (Germany). Fetal bovine serum (FBS), Dulbecco's Modified Eagle's Medium (DMEM), Trypsin/EDTA, and Penicillin–Streptomycin solutions were obtained from GIBCO (United Kingdom). Ethidium bromide, 9,10-phenanthrenequinone, and HPLC grade acetonitrile were from Merck (Germany). Rabbit polyclonal anti-Bax and anti-Bcl-2 antibodies, rabbit monoclonal anti-β-actin antibody, and rabbit horseradish peroxidase-conjugated secondary antibodies were obtained from Cell Signaling Technology (USA). Furthermore, enhanced chemiluminescence (ECL) western blotting detection reagents were from GE Healthcare. Flasks and petri dishes were purchased from Nunc (Denmark). Centricon® centrifugal filter devices (cut off: 3.5 kDa), low protein binding filter membranes (PVDF, 0.2 μm), and 24-Well Millicell hanging cell culture inserts (PET, 0.4 μm) were purchased from Millipore (France). All solutions were prepared in deionized water.

2.2. Preparation and purification of human glycated insulin

Human insulin was dissolved in 2 mM hydrochloric acid (HCl) at a final concentration of 1 mg/mL. In order to prepare the glycated insulin, 100 μg/mL of insulin was mixed with 220 mM D-glucose in sodium phosphate buffer (10 mM, pH 7.4) in the presence of the reducing agent, $NaBH_3CN$ (dissolved in 10 mM NaOH) for 24 h at 37 °C. Insulin without glucose was used as a control. Afterwards, the incubated mixture was acidified with 0.5 M acetic acid to stop the reaction and transferred into centricon® to remove free glucoses [26] followed by separation of glycated and non-glycated insulins by m-aminophenylboronic acid agarose affinity chromatography [30]. Following equilibration of the column with washing buffer (50 mM taurine/NaOH, pH 8.7, containing 20 mM $MgCl_2$), insulin samples were loaded. First, non-glycated insulin was eluted using washing buffer. Then, glycated insulin was eluted using the washing buffer plus 50 mM sorbitol [30]. In order to eliminate sorbitol and other molecules present in the eluted buffer, insulin and glycated insulin fractions were transferred into centrifugal centricons® and buffer exchanged to sodium phosphate buffer (10 mM, pH 7.4).

Furthermore, in order to determine the concentration of insulin and glycated insulin in the corresponding eluted fractions of affinity column, fluorescence of 9,10-phenanthrenequinone reagent was measured. In fact, 9,10-phenanthrenequinone reacts with arginine residues to form a fluorescent compound as an indication of protein concentration. Briefly, the two obtained eluates (100 μl) were mixed with 300 μl of 9, 10 phenanthrenequinone reagent (150 μM in ethanol) and 50 μl NaOH (2 N), and incubated for 3 h at 60 °C. Then, 450 μl HCl (1.2 N) was added and incubated in the dark at room temperature for 1 h. The fluorescence intensity was measured at excitation and emission wavelengths of 312 and 395 nm, respectively. Insulin solutions with concentrations in the range 0–20 μM were used for the standard curve [21]. The obtained glycated or non-glycated insulin solutions were sterilized by filtration through a sterile filter prior to cell treatment.

The purification of glycated insulin was confirmed employing a Shimadzu HPLC (LC-10A). The HPLC was equipped with an RP-8 column (4.6 × 250 mm, 5 μm; LiChrospher® 100) using UV detector. The analysis of 50 μg insulin and glycated insulin samples after being separated by affinity chromatography was performed using solvents A and B as the mobile phase. Solvent A consisted of 0.12% (v/v) TFA in water and solvent B was composed of 0.1% (v/v) TFA in 70% acetonitrile and 29.9% water. The concentration of acetonitrile increased in a linear gradient of 35% over 10 min, 56% over 20 min, and 70% over 5 min. The profiles were monitored at 206 nm [26,31].

The amount of glycation was determined using the fluorescamine assay according to the published procedure. Briefly, 495 μl of protein samples (10 μg/mL) were mixed with 5 μl fluorescamine reagent (3 mg/mL dissolved in DMSO) and incubated in the dark. Then, the fluorescence intensity was monitored at excitation and emission wavelengths of 390 and 486 nm, respectively. Both excitation and emission slits were set to 5 nm. In fact, free amino groups (primary amines) in proteins react with flourescamine reagent which causes it to be fluorescent. Therefore, the higher fluorsecamine emission means less glycation [21].

2.3. Conformational studies

The change induced in insulin conformation upon glycation was studied using absorbance spectra of insulin and glycated insulin (50 μg/mL) at the wavelength range of 250–350 nm on a UV-VIS spectrophotometer (Carry 100 Bio Varian) [32].

Intrinsic and extrinsic fluorescence measurements were performed on insulin samples after being modified through glycation to study the change in insulin's conformation. All fluorescence measurements were carried out on Cary-Eclipse fluorescence spectrophotometer (Varian) at 25 °C. Intrinsic fluorescence was determined using 5 μg/mL of insulin and glycated insulin. The emission spectra were recorded between 280 and 340 nm with an excitation wavelength of 276 nm [20]. The excitation and emission slits were set to 5 and 10 nm.

For extrinsic fluorescence studies, 250 μl of protein samples (50 μg/mL) were incubated with 50 μl ANS (3.3 mM in 20 mM Tris-HCl buffer)