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Research Paper

High glucose, glucose fluctuation and carbonyl stress enhance brain microvascular endothelial barrier dysfunction: Implications for diabetic cerebral microvasculature

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ABSTRACT

We previously demonstrated that in normal glucose (5 mM), methylglyoxal (MG, a model of carbonyl stress) induced brain microvascular endothelial cell (IHEC) dysfunction that was associated with occludin glycation and prevented by N-acetylcysteine (NAC). Herein, we investigated the impact of high glucose and low GSH, conditions that mimicked the diabetic state, on MG-induced IHEC dysfunction. MG-induced loss of transendothelial electrical resistance (TEER) was potentiated in IHECs cultured for 7 or 12 days in 25 mM glucose (hyperglycemia); moreover, barrier function remained disrupted 6 h after cell transfer to normal glucose media (acute glycemic fluctuation). Notably, basal occludin glycation was elevated under these glycemic states. TEER loss was exaggerated by inhibition of glutathione (GSH) synthesis and abrogated by NAC, which corresponded to GSH decreases and increases, respectively. Significantly, glyoxalase II activity was attenuated in hyperglycemic cells. Moreover, hyperglycemia and GSH inhibition increased MG accumulation, consistent with a compromised capacity for MG elimination. α-Oxoaldehydes (MG plus glyoxal) levels were elevated in streptozotocin-induced diabetic rat plasma. Immunohistochemistry revealed a prevalence of MG-positive, but fewer occludin-positive microvessels in the diabetic brain in vivo, and Western analysis confirmed an increase in MG–occludin adducts. These results provide the first evidence that hyperglycemia and acute glucose fluctuation promote MG–occludin formation and exacerbate brain microvascular endothelial dysfunction. Low occludin expression and high glycated-occludin contents in diabetic brain in vivo are factors that would contribute to the dysfunction of the cerebral microvasculature during diabetes.

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Introduction

Diabetes is a clinically important risk factor for cardiovascular and cerebrovascular diseases which are underscored by vascular endothelial dysfunction. It is well known that the diabetic condition is characterized by hyperglycemia and elevated plasma levels of reactive carbonyl species (RCS), but the mechanism by which RCS contribute to diabetes-associated cerebrovascular disease is poorly understood. Methylglyoxal (MG) is an RCS dicarbonyl metabolite precursor of advanced glycation endproducts, and is metabolized via a GSH-dependent glyoxalase detoxification pathway. Our recent results demonstrated that occludin glycation induced by MG disrupted barrier function in a human microvascular endothelial cell line (IHEC), and that N-acetylcysteine (NAC) afforded barrier preservation [1]. These results provide a compelling argument that the cerebral microvasculature in diabetes is sensitive to tissue levels of MG and glutathione (GSH).

The integrity of the cerebral microvasculature and microcirculation is maintained by the function of the blood–brain barrier (BBB) which reportedly is disrupted in diabetes [2]. The BBB phenotype is described by the neurovascular unit, comprising of brain capillary endothelial cells on the blood side and perivascular cells on the brain side of microvessels [3,4]. The BBB endothelial monolayer exhibits high transendothelial resistance that is conferred by the intercellular tight junctions between neighboring endothelial cells [5]. Occludin is a member of the tight junctional transmembrane proteins that regulates barrier electrical resistance

Abbreviations: AGEs, advanced glycation end products; BBB, blood–brain barrier; BSO, L-buthionine-(S,R)-sulfoximine; GSH, glutathione; HPLC, high-performance liquid chromatography; IHEC, immortalized human brain endothelial cell line; MG, methylglyoxal; NAC, N-acetyl-L-cysteine; PCA, perchloric acid; RCS, reactive carbonyl species; RIPA, radio immunoprecipitation assay buffer; SDL, S-D-lactoylglutathione; STZ, streptozotocin; TEER, transendothelial electrical resistance.

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and paracellular permeability [4]. We recently demonstrated that occludin is a target of MG glycation, and that MG-mediated occludin modification is associated with increased endothelial cell permeability in IHECs [1].

GSH was implicated to play a role in the BBB integrity [6]. The diabetic brain has been associated with decreased tissue GSH [7]; however, the mechanistic relationship between GSH decreases and cerebral microvascular dysfunction is unclear. We have found that GSH attenuated hyperglycemia- or MG-induced endothelial apoptosis [8,9] and barrier permeability [1]. These findings support a role for GSH in endothelial protection. With regards to MG handling, GSH is a rate-limiting cofactor in MG metabolism [11,12]. A reduction in cellular GSH levels leads to compromised glyoxalase function, resulting in the accumulation of free MG, and thereby increasing its glycating potential. Thus, GSH-dependent elimination of MG by glyoxalase I and glyoxalase II could be a major mechanism through which GSH protects against cerebral microvascular dysfunction in diabetes.

Collectively, MG-induced carbonyl stress (protein glycation or carbonylation) and endothelial GSH imbalance would potentiate cerebral microvascular dysfunction, and RCS-mediated dysfunction of the cerebrovascular endothelium and its pathology would be significant in diabetes. Importantly, given that triosephosphates derived from glucose metabolism is an MG source [12], the hyperglycemic state associated with diabetes would have an exacerbating role in brain endothelial injury. The current study addresses new links between hyperglycemia, MG and brain microvascular dysfunction. Using the previously established IHEC cell line, we sought to investigate whether states of high glucose or acute glucose fluctuation exacerbate MG-induced occludin glycation and barrier disruption, and if promoting GSH-dependent MG metabolism via exogenous NAC administration preserves barrier function. We further investigated if glycation of the brain microvasculature in vivo is a significant process during diabetes using a streptozotocin (STZ)-induced diabetic rat model. The results show that hyperglycemia and acute glycemic fluctuation potentiated MG-induced loss of IHEC barrier transendothelial electrical resistance (TEER), an event that was associated with increased occludin-MG adduct formation and prevented by NAC. Importantly, we found that diabetic rat brain microvessels exhibited decreased total occludin expression and elevated glycated–occludin adduct content.

Methods

Reagents

The following reagents were purchased from Sigma (St Louis, MO): D-glucose, methylglyoxal, N-acetyl-L-cysteine, L-buthionine-(S, R)-sulfoximine, insulin-transferrin sodium selenite solution, glutathione, D-lactate, D-lactic dehydrogenase, glutamic-pyruvate transaminase, S-D-lactoylglutathione, Medium 199, o-phenylenediamine, 2-methylquinoxaline, acetonitrile, and HRP-linked goat-anti-rabbit and goat-anti-mouse secondary antibodies. Anti-occludin rabbit polyclonal antibody was obtained from Invitrogen (Carlsbad, CA), anti-MG mouse monoclonal antibody from JcICA (Fukuroi, Japan), anti-actin mouse monoclonal antibody from BD Biosciences (San Jose, CA) and anti-GAPDH mouse monoclonal antibody from Santa Cruz (Santa Cruz, CA). HRP-linked goat-anti-rabbit and goat-anti-mouse antibodies, ECL, and chemi-luminescence detection reagents were purchased from Amersham Biopharmacia (Piscataway, NJ). Fetal bovine serum (FBS) was obtained from Atlanta Biologicals (Atlanta, GA). All other chemicals were of reagent grade and were purchased from Sigma or local sources.

Cell culture and cell incubations

The human brain microvascular endothelial cell line (IHEC) was provided by Dr. Danica Stanimirovic of the National Research Council Canada's Institute for Biological Sciences and was maintained by Dr. Steve Alexander at LSU Health Sciences Center. Routinely, IHECs were cultured in M-199 medium containing 5 mM glucose (normal glucose), 10% FBS, 1% insulin–transferrin–sodium selenite solution, and 1 × antibiotic/antimycotic at 37 °C in 5% CO_2 on T-75 flask. In all cell studies, IHECs were seeded at specific densities 5 days before the experiment. High glucose-adapted IHECs were achieved by culturing cells in 25 mM glucose for 7 or 12 days. Acute glycemic fluctuation was achieved by transferring 25 mM glucose-adapted cells (12 days) to normal glucose media for 6 h (designated 25→5 mM glucose).

IHECs (1.5×10^4) were cultured on 8-μm inserts in 24-well plates for measurements of TEER. For assays of GSH, Western blot and glyoxalase I/II activities, IHECs (0.4×10^6) were grown in 6-well plates. All experiments were conducted on confluent cell monolayers (5 days post-seeding). The experimental glucose concentrations include: 5 mM (normoglycemia), 25 mM (hyperglycemia), or 25→5 mM glucose (acute glycemic fluctuation). To achieve high or low cellular GSH, IHECs were pretreated overnight with 1 mM NAC or 50 μM BSO, respectively. After washing, 2 mM NAC or 300 μM BSO were added to the incubation to maintain elevated or low GSH status in these cells throughout the experiment. A range of MG concentrations were used depending on the type of experiments. MG concentrations of 50 μM to 1 mM were used to examine the time course and dose dependency of TEER responses under normal and high glucose states. These MG levels were previously employed in our recent studies [1]. Since high glucose will contribute to MG production, a lower dose of 300 μM MG was then used in subsequent experiments to test the effect of BSO and NAC under hyperglycemic conditions. An MG level of 50 μM MG was near physiological in diabetic rat plasma (see Fig. 6).

Measurement of transendothelial electrical resistance (TEER)

Studies of TEER were carried out in HBSS containing 25 mM HEPES and 10% FBS (pH 7.35) at 37 °C and 5% CO_2. After an initial 1 h adjustment period, changes in electrical resistance at different concentrations of MG without or with NAC or BSO (as above) were recorded for up to 10 h using an epithelial voltohmeter (EVOM, World Precision Instruments, Sarasota, FL). Only inserts with a minimum baseline monolayer resistance of 200 Ω/0.33 cm^2 were used. TEER was expressed as the percent of the baseline to account for batch-to-batch variation.

Western analyses of occludin and MG–occludin

Cell extracts

Cells were harvested and lysed in RIPA buffer containing 50 mM Tris, pH 7.4, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100 and a cocktail of protease inhibitors, viz., aprotinin, PMSF, okadaic acid, and leupeptin.

Tissue extracts

Cerebral vessels were isolated as described previously [13]. Briefly, the brain was removed from the skull and pia matter containing large vessels was gently teased off and placed in cold PBS. The brain tissue was then homogenized in ice-cold PBS (polytron, 1 min). The homogenate was passed through an 18G needle 10 times, and centrifuged at 2000*g* for 10 min at 4 °C. The pellet was suspended in ice-cold PBS, gently layered on top of 30 ml of 15% dextran (MW 38,400) and centrifuged at 17,400*g* for

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