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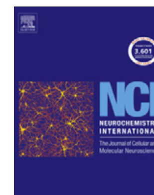

The unfolded protein response to endoplasmic reticulum stress in cultured astrocytes and rat brain during experimental diabetes

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ABSTRACT

Oxidative-nitrosative stress and inflammatory responses are associated with endoplasmic reticulum (ER) stress in diabetic retinopathy, raising the possibility that disturbances in ER protein processing may contribute to CNS dysfunction in diabetics. Upregulation of the unfolded protein response (UPR) is a homeostatic response to accumulation of abnormal proteins in the ER, and the present study tested the hypothesis that the UPR is upregulated in two models for diabetes, cultured astrocytes grown in 25 mmol/L glucose for up to 4 weeks and brain of streptozotocin (STZ)-treated rats with diabetes for 1–7 months. Markers associated with translational blockade (phospho-eIF2α and apoptosis (CHOP), inflammatory response (inducible nitric oxide synthase, iNOS), and nitrosative stress (nuclear translocation of glyceraldehyde-3-phosphate dehydrogenase, GAPDH) were not detected in either model. Nrf2 was present in nuclei of low- and high-glucose cultures, consistent with oxidative stress. Astrocytic ATF4 expression was not altered by culture glucose concentration, whereas phospho-IRE and ATF6 levels were higher in low- compared with high-glucose cultures. The glucose-regulated chaperones, GRP78 and GRP94, were also expressed at higher levels in low- than high-glucose cultures, probably due to recurrent glucose depletion between feeding cycles. In STZ-rat cerebral cortex, ATF4 level was transiently reduced at 4 months, and p-IRE levels were transiently elevated at 3 months. However, GRP78 and GRP94 expression was not upregulated, and iNOS, amyloid-β, and nuclear accumulation of GAPDH were not evident in STZ-diabetic brain. High-glucose cultured astrocytes and STZ-diabetic brain are relatively resistant to diabetes-induced ER stress, in sharp contrast with cultured retinal Müller cells and diabetic rodent retina.

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1. Introduction

Diabetic complications affect many peripheral organ systems (Brownlee, 2005; Giacco and Brownlee, 2010), and, although the central nervous system (CNS) is considered to be relatively resistant to debilitating effects of diabetes, accumulating evidence supports progressive neurological and cognitive dysfunction in animals and humans with Type I or Type II diabetes (McCall, 2002, 2004; Kodl and Seaquist, 2008; Wrighten et al., 2009; Reijmer et al., 2010; Sima, 2010; Jacobson et al., 2011; Strachan et al., 2011; Reagan, 2012). Furthermore, recurrent hypoglycemic episodes arising from strict glycemic control contribute to decrements in brain function and cellular metabolism (Jiang et al., 2009; McNay and Cotero, 2010). Oxidative stress and inflammatory responses are components of the pathophysiology of diabetes in many tissues (Giacco and Brownlee, 2010) and may contribute to altered brain function. For example, levels of reactive oxygen/nitrogen species (ROS/RNS) are elevated in streptozotocin (STZ)-diabetic rat brain and in cultured astrocytes grown in high (25 mmol/L) glucose, and increased oxidative stress precedes the onset of reduced gap junctional communication among astrocytes by several days (Gandhi et al., 2010). Three of our findings impli-

Abbreviations: ATF4, activating transcription factor 4 (also designated as CREB-2, cAMP response element (CRE) binding protein2); ATF6, activating transcription factor 6; BSA, bovine serum albumin; CHOP, CCAAT/enhancer-binding protein-homologous protein; CNS, central nervous system; DMEM, Dulbecco's modified Eagle's medium; eIF2α, eukaryotic translation initiation factor 2α; ER, endoplasmic reticulum; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; glc, glucose; GRP, glucose-regulated protein; GRP78 or GRP94, glucose-regulated protein, molecular weight 78 or 94 kDa, respectively; iNOS/NOS2, inducible nitric oxide synthase; IRE1α, inositol-requiring enzyme 1α; KDEL, tetrapeptide sequence located at the carboxy-terminal of luminal ER proteins that serves as retrieval motif for retention in the ER (the antibody against the sequence SEKDEL in GRP78 detects both GRP78 and GRP94); LPS, lipopolysaccharides; NO, nitric oxide; PBS, phosphate-buffered saline; PBS-T, PBS-Tween-20; PTX, PBS triton X; PDI, protein disulfide isomerase; p-eIF2α, phospho-initiation factor 2α; PERK, protein kinase (PRK)-like ER kinase; p-IRE, phospho-IRE; p-PERK, phospho-PERK; ROS, reactive oxygen species; RNS, reactive nitrogen species; SNP, sodium nitroprusside; STZ, streptozotocin; UPR, unfolded protein response.

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cate oxidative-nitrosative and ER stresses as elements underlying disruption of communication among astrocytes in experimental diabetes, (i) treatment of low-glucose cultures with a nitric oxide (NO) donor or tunicamycin (to evoke endoplasmic reticulum (ER) stress) is sufficient to impair gap junctional dye transfer, (ii) the decrement in high-glucose cultured astrocytes is rescued by a reducing agent, and (iii) pharmacological treatments that protect or rescue gap junctional communication include a superoxide dismutase mimetic, an inhibitor of nitric oxide synthase (NOS), and small molecules that facilitate protein folding (Gandhi et al., 2010; Ball et al., 2011).

ER stress arises when protein folding capacity is inadequate due to increased rates of synthesis or accumulation of misfolded or abnormal proteins, and it activates the unfolded protein response (UPR). The UPR governs preferential translation of chaperone proteins that facilitate protein folding and it triggers apoptosis when severe. The UPR has three branches that govern translational control and chaperone protein expression, PERK (PRK-like protein kinase), IRE-1 (inositol-requiring enzyme-1), and ATF6 (activating transcription factor-6) (Ron and Walter, 2007; Kaufman et al., 2010; Walter and Ron, 2011; Back and Kaufman, 2012; Ozcan and Tabas, 2012). These three sensor proteins interact with the protein-folding chaperone GRP78 (glucose-regulated protein-78 or BiP) in the ER, and when misfolded proteins accumulate GRP78 associates with these proteins, activating the UPR. ER stress occurs in subjects with diabetes (Eizirik et al., 2008; Volchuk and Ron, 2010; Back and Kaufman, 2012), with high fat diet, during insulin resistance, and in Type 2 diabetes (Ozcan et al., 2004, 2006), in brain during neurodegenerative disease (Ozcan and Tabas, 2012), and in diabetic retina and cultured retinal Müller cells grown in high glucose (Oshitari et al., 2008; Jing et al., 2012; Zhong et al., 2012).

Cultured cells from many tissues are commonly used as models for diabetes, and we used comparative studies in cultured astrocytes and diabetic rat brain slices to evaluate gap junctional trafficking. This approach facilitated mechanistic studies that minimized animal use and identified potential therapeutic agents that can be tested in vivo in diabetic animals (Gandhi et al., 2010). We also found that levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and actin were differentially altered in diabetic astrocytes and rat brain (Ball et al., 2011), indicating that two proteins commonly-used as loading controls in Western blots are not stable reference proteins in diabetes. GAPDH is a multifunctional cytoplasmic glycolytic enzyme that is regulated, in part, by nitric oxide. S-nitrosylation of GAPDH inhibits its enzymatic activity and triggers nuclear translocation where it can initiate apoptosis (Molina y Vedia et al., 1992; Padgett and Whorton, 1995; Mohr et al., 1996; Hara et al., 2006; Tristan et al., 2011). Nuclear accumulation of GAPDH occurs during experimental diabetes in cultured Müller cells and rat retina (Kusner et al., 2004; Kanwar and Kowluru, 2009), and 48 h exposure of Müller cells to 25 mmol/L glucose is sufficient to increase GAPDH nuclear translocation, followed by apoptosis at four days (Kusner et al., 2004; Yego et al., 2009; Yego and Mohr, 2010). An immortalized rat retinal capillary endothelial cell line is also very sensitive to short-duration (8 or 16 h) exposure to 25 mmol/L glucose and exhibits UPR and inflammatory responses (Chen et al., 2012). Thus, nitrosative and ER stresses are linked to diabetic retinopathy. The objective of this study was to (i) test our hypothesis that the UPR is upregulated in high-glucose astrocyte cultures and STZ-diabetic rat brain, and (ii) if a strong UPR response occurs in diabetic astrocytes or STZ-rat brain, evaluate effects of our previously-identified therapeutic agents that prevented or rescued the acquired deficits in gap junctional communication. The results demonstrate that cultured astrocytes and STZ-diabetic rat brain are relatively resistant to ER stress.

2. Materials and methods

2.1. Reagents

Low glucose (5.5 mmol/L, #12320-032) and high glucose (25 mmol/L, #12430-054) Dulbecco's modified Eagle's medium (DMEM), Dulbecco's phosphate-buffered saline (PBS) containing $CaCl_2$ and $MgCl_2$ (Gibco #14040), penicillin, streptomycin, amphotericin B, Hoechst dye (#33258), and trypsin were from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was from Hyclone (Logan, UT), Complete Protease Inhibitor cocktail tablet (#04693 116 001) from Roche Diagnostics GmbH (Mannheim, Germany), and sodium fluoride from ICN (K&K Laboratories, Carlsbad, CA). Dibutyryl cAMP (dBcAMP), L-leucine methyl ester hydrochloride (L-LME), poly-d-lysine, streptozotocin (STZ; N-(methyl-nitrosocarbamoyl-α-D-glucosamine), sodium nitroprusside (SNP), N-[4-[1-(3-Aminopropyl)-2-hydroxy-2-nitrosohydrazino]butyl]-1,3-propanediamine (Spermine NONOate), thapsigargin, tunicamycin, lipopolysaccharides (LPS) from E. coli 055:B5 (#L-2880), Griess reagent (modified, #G4410), 2- mercaptoethanol, Na orthovanadate, neocuproine, bovine serum albumin (BSA, #A9647-100), and Na azide (#S8032) were from Sigma–Aldrich (St. Louis, MO). The albumin standard (#2309), Pierce BCA Protein Assay Reagent A (#23228), and Pierce BCA Protein Assay Reagent B (#1859078) were from Thermo Fisher Scientific (Rockford, IL). Hepes, Triton-X-100, Tween-20, SDS electrophoresis grade, paraformaldehyde, Tissue Protein Extraction Reagent (T-PER, #78510), Tris Base (#BP152-1), glycine tissue culture grade (#BP381-1), sodium chloride (#S271-1), sodium phosphate monobasic (#BP329-500), and sodium phosphate dibasic (#S374-500) were from Fisher Scientific (Pittsburgh, PA). The 4X Protein Loading Buffer (#928-40004), Odyssey One-Color Molecular Weight Markers (#928-40000), and Odyssey Blocking Buffer (#927-40003) were purchased from Li-Cor (Lincoln, NE).

2.2. Antibodies

Primary antibodies used to assess the UPR were as follows: goat-anti-PERK (#sc-9477, Santa Cruz Biotechnology, Santa Cruz, CA, 1:500), rabbit-anti-phospho-PERK (#3179, Cell Signaling Technology, Danvers, MA, 1:1000), goat-anti-eIF2α (#sc-30882, Santa Cruz, 1:1,000), rabbit-anti-phospho-eIF2α (#9721, Cell Signaling, 1:1000), rabbit-anti-ATF4/CREB-2 (#sc-200, Santa Cruz, 1:600), goat-anti-IRE1 (#ab11544, AbCam, Cambridge, MA, 1:1000), rabbit-anti-phospho-IRE1 (#48187, AbCam, 1:1,000), mouse-anti-CHOP (#2895, Cell Signaling, 1:500), mouse-anti-ATF6 (#IMG-273, Imgenex, San Diego, CA, 1:500), rabbit-anti-GRP78 (#sc-13968, Santa Cruz, 1:500), and mouse-anti-KDEL (10C3, #ADI-SPA-827, Stressgen, Enzo Life Sciences International, Plymouth Meeting, PA, 1:1,000). Primary antibodies used to evaluate other aspects of cellular stress responses (see below) were: rabbit-anti-protein disulfide isomerase (PDI, #2446, Cell Signaling), goat polyclonal anti-iNOS/NOS2 (#sc-650G, Santa Cruz), rabbit anti-GAPDH (#G9545, Sigma, 1:25,000 on blots), rabbit anti-amyloid-β_{1-40} (#A8326, Sigma), mouse monoclonal anti-β-amyloid (4G8, Sig-39220 (Signet/Covance Inc, Princeton, NJ), and rabbit polyclonal anti-Nrf2 (H-300, #sc-13032, Santa Cruz). Secondary antibodies diluted in Odyssey blocking buffer were IRDye 800CW Donkey anti-goat (#926-32214, Li-Cor, 1:30,000), IRDye 800CW Donkey anti-mouse (#926-32212, Li-Cor, 1:15,000), and IRDye 680LT Donkey anti-rabbit (#926-68023, Li-Cor, 1:20,000). Secondary antibodies used for cell staining, goat anti-rabbit IgG Texas Red (#T6391), goat anti-mouse IgG Texas Red (#T862), and rabbit anti-goat IgG Alexa 594, were from Invitrogen/Life Technologies (Grand Island, NY). Additional assays were carried out using

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