Published in final edited form as:

Neurochem Int. 2013 April ; 62(5): 784–795. doi:10.1016/j.neuint.2013.02.009.

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-eIF2 α) 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.

Keywords

astrocyte; gap junction; glucose; neuron; oxidative stress; streptozotocin

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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; Wrihten 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 implicate 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; Ozcan et al., 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 48h 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 16h) 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 CaCl₂ and MgCl₂ (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 (#92840000), 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-eIF2 α (#sc-30882, Santa Cruz, 1:1,000), rabbit-anti-phospho-eIF2 α (#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 Chemiglow detection and a BioRad ChemiDoc XRS system with Image Lab software (2.0.1).

2.3 Astrocyte cell culture

Astrocytes were prepared from cerebral cortex of newborn Wistar-Hanover rats as described (Ball et al., 2011). In brief, astrocytes were grown in DMEM containing 5.5 mmol/L glucose, 10% (v/v) fetal bovine serum, 100 units of penicillin, 100 mg/ml of streptomycin, and L-LME (to reduce microglia (Esen et al., 2007)) at 37°C in humidified air containing 5% CO₂, and the medium was changed every 3-4 days. After 2 weeks, confluent cells were trypsinized and re-plated in poly-D-lysine-coated plates or glass cover slips in low-glucose medium plus a fungicide amphotericin B (2.5 µg/mL). At confluence differentiation was induced (0.25 mmol/L dBcAMP), and cells were maintained in medium with 0.25 mmol/L dBcAMP, amphotericin, and 5.5 or 25 mmol/L glucose for an additional 3 (n = 2 batches), 7 (n = 3 batches), 14 (n = 3 batches), 21 (n = 3 batches), or 28 days (n = 1 batch) days. Each independent culture batch was from a different litter and each batch consisted of parallel cultures grown in high or low glucose. Some batches were also assayed at 1, 2, or 17–20h and 1 or 2 weeks after transfer to high glucose; others were grown without cAMP treatment.

Positive controls for ER stress were generated by treating 14-day astrocyte cultures with compounds that induce ER stress, tunicamycin (a glycosylation inhibitor; 10 µg/ml for 20 h) or thapsigargin (an ER calcium pump inhibitor; 1 µg/ml for 3, 5, 7, or 18 h) (Kass and Orrenius, 1999; Kaufman, 1999). Sodium nitroprusside (SNP) was used to produce nitrosative stress (100, 200, 400, or 800 µmol/L for 1–48h). Lipopolysaccharide (LPS, 1 or 2 µg/mL for 48h, or as indicated) was used to try to upregulate inducible nitric oxide synthase (iNOS), nitrite accumulation, and GAPDH nuclear translocation. Nitrite levels in culture media were assayed with the Griess reagent (#G4410, Sigma) as described by the manufacturer.

Astrocyte were initially extracted with lysis buffer (in mmol/L: 25 Hepes, 50 NaCl, 10 NaF, 1 Na₃VO₄, 0.1 neocuproine, 1 Complete Protease Inhibitor tablet, 1% Triton-X-100, and 0.1% SDS) modified from that of Forrester et al. (2007; 2009), which is used for biotin switch assays of S-nitrosylated proteins. Subsequent samples were extracted with lysis buffer containing 150 mmol/L NaCl (see Results). Lysates were centrifuged and stored at –80°C.

2.4 Streptozotocin (STZ)-induced diabetes

Non-fasted male Sprague-Dawley rats (200–300 g; Harlan, Indianapolis, IN) were given STZ (in 33 mmol/L citrate-buffered saline, pH 4.5; 65 mg/kg body weight i.p.) or the same volume of vehicle (Romanovsky et al., 2006). Diabetic rats were identified by high blood glucose levels on day 3 after STZ. Brains from diabetics (n = 5/group, except 5 mo., n = 3) and age-matched, vehicle-injected controls (n = 4/group, except 5 mo., n = 3) were harvested at 1, 2, 3, 4, 5, and 7 months. Tissue was homogenized in ice-cold T-PER reagent (in mmol/L: 50 NaF, 1 Na₃VO₄, 0.1 neocuproine, T-PER, and 1 Complete Protease Inhibitor tablet), centrifuged, and stored at –80°C. All animal use procedures were in strict

accordance with the NIH Guide for Care and Use of Laboratory Animals and were approved by the local Animal Care and Use Committee. Efforts were made to minimize animal suffering and cultured astrocytes were used as an alternative to in vivo techniques to reduce the number of animals.

2.5 Glucose, lactate, and protein assays

Lactate and glucose levels were measured with a Model 2700 Dual Channel Analyzer (Yellow Springs Instruments, Yellow Springs, OH) in samples taken at timed intervals after culture medium change and in plasma samples from STZ-diabetic and age-matched, vehicle-treated control rats. Lysate protein contents were assayed in triplicate using the Pierce[®] bicinchoninic acid (BCA) protein assay using bovine serum albumin as the standard.

2.6 Polyacrylamide gel electrophoresis and Western blotting

Extracts were mixed with Odyssey loading buffer containing 10% (v/v) mercaptoethanol, and proteins were separated on TGX pre-cast 10% polyacrylamide gels (#456-1033, BioRad, Hercules, CA), transferred onto Odyssey nitrocellulose membranes, blocked, incubated with primary antibody overnight, then the secondary antibody, and imaged with a Li-Cor Odyssey Infrared Imaging System. Optimization studies (not shown) were used to determine dilutions for the primary and secondary antibodies, the composition of the blocking reagent that minimized non-specific bands (5% BSA in Tris-buffered saline containing Tween-20 (TBS-T; 0.5 mol/L NaCl, 20 mmol/L Tris, 0.05% (v/v) Tween-20, pH 7.5) for eIF2 α , ATF4, and CHOP, and Odyssey blocking buffer + 0.1% Tween 20 for the others), antibody specificity, and linearity of Western blot signals. Levels of CHOP, p-eIF2 α , iNOS, PDI, PERK, and p-PERK were near or below the limits of detection in extracts from high and low glucose astrocyte cultures and cerebral cortex from control and STZ-diabetic rats. Linear responses over the range of at least 5 to 40 μ g protein loads were obtained for ATF6, ATF4, eIF2 α , p-IRE, GRP78, and KDEL (GRP78 and GRP94), and subsequent comparisons were made using 20 μ g protein loads so 2-fold increases or decreases in protein amount would fall within the linear ranges. Preincubation of anti-ATF4 and anti-p-IRE1 antibodies with their commercially-available blocking peptides eliminated the prominent bands at the expected molecular weight (data not shown).

'Loading' controls are used to normalize data and minimize blot-to-blot variability. The signal for a protein of interest is divided by that of a reference ('housekeeping') protein in the same sample lane, and these ratios are used for comparisons with samples assayed on different gels. This approach requires that the level of the internal standard protein is not altered by experimental conditions. Concerns regarding loading controls discussed in our previous study (Ball et al., 2011) and in the cited references include the following. (i) Proteins of interest are often present in low quantities whereas reference proteins are generally higher abundance, and optimization of protein load for detection of low-abundance proteins may cause overloading of the reference protein. If the reference protein signals are near or beyond the linear response range, small signal changes can correspond to large differences in protein load and corrections are insensitive to variations in load or blot transfer. (ii) Levels of commonly-used reference proteins do change during development and disease. (iii) Proof of stability of a reference protein requires its calibration against a known standard.

We, therefore, developed and validated an alternative procedure in which signals from proteins of interest in experimental samples are normalized to those of the same protein in control samples assayed at the same protein load in the same gel and blot, i.e., the sensitivity and linear range of the signal are the same for the protein of interest in each experimental condition (Ball et al., 2011). Thus, when the experimental/control ratio is calculated, protein

amount cancels out, as would the signal for a loading control if its value were identical for both samples, i.e., $[(\text{signal for protein in a high glucose sample} / \mu\text{g protein loaded})] / [(\text{signal for protein in a low glucose sample} / \mu\text{g protein loaded})]$. We demonstrated that (i) blots of replicate samples loaded on the same gel are reproducible (coefficient of variation, CV = 8%), indicating that within-blot variations arising from loading and transfer were minor; (ii) replicates of ratios for pairs of samples assayed on different gels for connexin (Cx) proteins, Cx30 and Cx43, are reproducible (CV = 2–11%), whereas ratios for actin and GAPDH were more variable; and (iii) levels of commonly-used ‘housekeeping’ reference proteins, GAPDH and actin, change in diabetic samples. This approach was used in the present study to evaluate changes in the levels of proteins of interest in experimental diabetes. For each culture batch, samples for each time point for the high and low glucose cultures were assayed using the same total protein load on the same gel and blot for each protein of interest. To minimize ratio variability and account for the possibility of transfer differences among lanes of the same blot, each sample was assayed in duplicate, some in triplicate; the occasional spurious results were discarded and assays repeated. Mean values for each batch were used to calculate the mean and SD for all batches for a given time point. For interest, 3- and 28-day data are reported, but these time points had one or two batches, and these data were not used for statistical analysis. For samples from STZ-diabetic ($n = 5/\text{group}$) and age-matched, vehicle injected control ($n = 4/\text{group}$) rat brain, the STZ/control ratios were calculated by normalizing the value for each protein from each STZ rat to the mean signal intensity for that protein for all controls assayed on the same blot. The ratios for each sample were averaged for the duplicate gels, and the mean ratio and SD for the diabetic rats calculated for each time point.

2.7 Immunofluorescence staining of cultured astrocytes and rat brain tissue

Cells were fixed with 2% paraformaldehyde in 0.1 M PBS, pH 7.4, except for assays of nuclear translocation of GAPDH or Nrf2, when cells were fixed as described by (Takahashi et al., 2012). Fixed cells were blocked with 10% goat serum, incubated with anti-GAPDH (1:200, in 10% goat serum in PTX), anti-Nrf2 (1:100), or anti-iNOS (1:100), followed by a Texas Red- or Alexa 594-labeled secondary antibody (1:500), and nuclei were stained with Hoechst’s dye solution. Frozen brain sections (10 μm -thick) were immunostained as described (Ball et al., 2007; Ball et al., 2010). They were blocked, incubated with goat anti-iNOS (1:50, 1:100, or 1:200), rabbit anti-GRP78 (1:50, 1:100, 1:200, or 1:400), mouse anti-KDEL (1:100), rabbit anti-amyloid-beta_{1–40} (1:100), mouse anti- β -amyloid (1:600), and rabbit anti-GAPDH (1:200), followed by a Texas Red- or Alexa 594-conjugated secondary antibody (1:500). Cell and tissue samples were examined with a Zeiss Axioskop 2 microscope (Carl Zeiss, Germany), and images were captured with a MTI CCD 72 camera (Dage-MTI, Michigan City, IN) and processed with MCID Imaging Software (InterFocus Imaging, Cambridge, UK). Selected samples were also analyzed by confocal microscopy using a Nikon C1-Plus system and Nikon E600FN microscope and NIS-elements software (Nikon Instruments, Melville, NY).

2.8 Statistics

Mean ratios (and SDs) were compared to 1.0 using the one-sample t-test, i.e., if the ratio is one, the values for each group are not different. Comparisons among two groups were made with the t-test, and among 3 or more groups with ANOVA and posthoc test. Analyses were performed with GraphPad Prism® software, version 5.04. $P < 0.05$ was considered significant.

3. Results

3.1. Expression of UPR proteins in astrocytes cultured in low or high glucose

Our initial series of experiments with cultured astrocytes to assess temporal profiles of ER stress protein expression after culture in low and high glucose for 3, 7, 14, 21, and 28 days used a lysis buffer based on that of Forrester et al. (2007; 2009), which contained 50 mmol/L NaCl and is used to extract cells for biotin switch assays of S-nitrosylated proteins. A prominent immunoreactive band was detected at ~80 kDa with anti-ATF4 for samples from high and low glucose cultures and thapsigargin-treated low glucose cultures (Fig. 1A-a, left panel), and this and other bands were eliminated by pre-adsorption of the antibody with the blocking peptide (not shown). However, the molecular weight for ATF4 is in the range of ~40–55 kDa (e.g., (Kumar et al., 2003; Begum et al., 2012; Chen et al., 2012)), and when the composition of the lysis buffer was compared with other extraction buffers, the NaCl concentration was lower than the commonly-used 150 mmol/L. ATF4 is a transcription factor that forms heterodimers with other basic-region leucine zipper (bZip) proteins (for examples, see (Murphy and Kolstø, 2000; Hai and Hartman, 2001; Kilberg et al., 2009) and references cited therein), raising the possibility that the low NaCl concentration may not be sufficient to separate binding partners. This notion is supported by an increase in the intensity of the ~55 kDa band in all samples when the NaCl contents of low-salt extracts were increased to 150 mmol/L prior to electrophoresis; signal intensity was highest in thapsigargin-treated samples (Fig. 1A-a, right panel). Further increases in levels of NaCl, mercaptoethanol, deoxycholate, triton, or NaH₂PO₄ in the extracts prior to electrophoresis did not completely eliminate the cross-reactive proteins labeled by anti-ATF4, and increasing the NaCl concentration from 50 to 150 mmol/L did not alter the molecular weights of proteins detected by anti-p-IRE and anti-ATF6 (not shown). The high-to-low glucose ratios for the ~80 kDa band derived from the 50 mmol/L NaCl extracts over the interval from 3–21 days were not significantly different from 1.0 (not shown), and a ratio of 1.03 was obtained for three additional independent batches of 14-day cultures extracted with lysis buffer containing 150 mmol/L NaCl (Fig. 1A-b).

The level of p-IRE increased in thapsigargin-treated astrocyte cultures (Fig. 1B-a) and it was higher in low compared with high glucose cultures (Fig. 1B-b). The expression level was, however, very low and near the limit of detection.

Cleaved ATF6 is reported to be ~50 kDa glycosylated protein (Haze et al., 1999; Kumar et al., 2003; Teske et al., 2011) and ~36 kDa forms have also been reported (Mao et al., 2007; Liu et al., 2008; Jeyasuria et al., 2011; Kumashiro et al., 2011), as observed in the present study (Fig. 1C-a). Protein levels of the cleaved ATF6 tended to be somewhat higher in the low glucose compared with high glucose cultures, but the ratios were only statistically significantly different from 1.0 when sample means for all time points were pooled (Fig. 1C-b).

Levels of GRP78 and GRP94 were assayed with the KDEL antibody that detects both proteins in tunicamycin-treated positive controls (Fig. 2A). GRP78 and GRP94 were assayed in duplicate for all samples, and their levels were very low and variable with respect to culture duration and among independent culture batches, as illustrated for one batch (Fig. 2B). GRP78 was present in low glucose cultures, but it was close to or below the limit of detection in high-glucose cultures (Fig. 2B, left panel); high/low glucose ratios were, therefore, not calculated. Levels of GRP94 were also much higher in the low-compared with high-glucose cultures at all time points (Fig. 2B, right panel). The GRP94 signal intensity always exceeded that for GRP78 at each time point in all cultures.

GRP78 and GRP94 are known to be upregulated by glucose starvation and other stress conditions (Shiu et al., 1977; Lee, 2001), raising the possibility that faster depletion of glucose from the low-glucose culture medium may underlie upregulation of these two glucoseregulated proteins. Indeed, nearly all of the glucose in the low-glucose medium was consumed within 24 h (Fig. 2C, left panel), whereas only about half of the glucose was metabolized at the same time in the high-glucose cultures (Fig. 2C right panel). At 96 h after feeding, the medium glucose level in the high-glucose cultures still exceeded 5 mmol/L, which approximates the initial level in the low-glucose cultures. Both cultures quickly converted glucose to lactate that was released to the culture medium. After the medium glucose was exhausted, the lactate level in the low-glucose culture medium progressively fell with time, consistent with its oxidative metabolism by the astrocytes. Thus, after each routine feeding that took place at 3–4 day intervals, the low-glucose cultures cycled between glycolytic metabolism of glucose followed by oxidative metabolism of lactate. In the high-glucose cultures, the maximal medium lactate was twice that in the low glucose cultures, peaking at 24.4 ± 1.4 mmol/L at 72h, then falling to 19.3 ± 0.9 mmol/L at 96 h ($p < 0.05$; ANOVA, Tukey's test) (Fig. 2C, right panel), indicating that the high glucose cultures oxidized lactate in the presence of 6 mmol/L glucose. However, the high medium lactate levels did not prevent glucose utilization by the astrocytes, even when lactate levels exceeded 6–12 mmol/L, and glucose was consumed throughout the entire 96h interval (Fig. 2C, right panel).

3.3. iNOS and nitrite levels in low and high glucose and LPS-treated astrocyte cultures

Experimental diabetes is associated with induction of iNOS in peripheral tissues, retina, and brain, and inflammatory processes are thought to contribute to diabetic pathophysiology (e.g., (Nagareddy et al., 2005; Kern, 2007; Liu and He, 2010; Nawa et al., 2010; Cai et al., 2011; Chen et al., 2012)). iNOS was not detectable in cultured astrocytes grown in either low and high glucose for up to 28 days, whether assayed by immunofluorescent staining of fixed cells or in Western blots of cell extracts (data not shown). Nitrite levels in the culture medium from astrocytes grown for 3, 24, 48, 53h, or 96h in either low or high glucose were below the limit of detection (<0.04 $\mu\text{mol/L}$), consistent with low NOS expression and activity in these astrocytes.

Lipopolysaccharide (LPS) treatment was used in an attempt to upregulate iNOS expression as a positive control for the iNOS and nitrite assays because treatment of cultured astrocytes with LPS (1 $\mu\text{g/mL}$) induces mRNA expression for iNOS and glucose-6-phosphate dehydrogenase, enhances medium nitrite accumulation (to ~ 12 – 16 $\mu\text{mol/L}$ at 24–48h), and increases flux through the pentose phosphate pathway to generate more NADPH to defend against oxidative damage ((Garcia-Nogales et al., 1999) and cited references). However, LPS (2 $\mu\text{g/mL}$ for 24h) evoked only small increases in medium nitrite levels (1.7, 2.9, and 5.1 $\mu\text{mol/L}$ in three experiments), and additional experiments using 1, 3, 6, 12, or 24 $\mu\text{g/mL}$ LPS for 24h also yielded low medium nitrite levels (<3 $\mu\text{mol/L}$). Although L-LME and shaking treatments (see Methods) remove nearly all microglia from our astrocyte cultures, the few microglia that remain could be visualized as iNOS-positive after LPS treatment, thereby serving as a positive control for the iNOS-negative astrocytes in the same cultures.

Amphotericin B enhances the cytokine-evoked activation of iNOS and nitrite production (Trajkovic et al., 2001), raising the possibility that culture conditions influence the astrocytic inflammatory response. This possibility was evaluated in experiments in which one batch of astrocytes grown in low glucose was treated with cAMP without amphotericin B and another was treated with amphotericin B without cAMP; the respective medium nitrite levels were 0.6 and 4.6 $\mu\text{mol/L}$, suggesting that amphotericin B may stimulate NO production. Also, amphotericin-treated astrocytes grown in the presence or absence of cAMP in either high or low glucose consumed more glucose compared with parallel cultures

without amphotericin. These findings raise the possibility that flux through the pentose phosphate pathway is enhanced in amphotericin-treated cultures due to increased defensive action against reactive oxygen-nitrogen species, thereby stimulating total glucose utilization in these cultures. Follow-up of these intriguing preliminary data is of interest, but beyond the scope of the current study. Taken together, the above data showing low iNOS expression and low nitrite accumulation in our cultured astrocytes are consistent with results of two studies in which cultured astrocytes did not upregulate iNOS mRNA, iNOS immunostaining, or medium nitrite level unless they were treated with cytokines (Trajkovic et al., 2001) or LPS (1 mg/mL) plus interleukin 1 beta, interferon gamma, and tumor necrosis factor alpha (Kuegler et al., 2012).

3.4. Localization of GAPDH and Nrf2 in high and low glucose astrocyte cultures

Exposure of cultured retinal Müller cells to 25 mmol/L glucose for 1–4 days causes nuclear accumulation of GAPDH, which is associated with apoptosis and diabetic retinopathy (see Introduction). However, growth of astrocytes in high glucose for 1–2h, 18h, or 2–14 days in the present study did not increase nuclear localization of GAPDH compared with low glucose cultures, as illustrated for representative cultures in Fig. 3A. GAPDH labeling was highest in the cytoplasm and processes, whereas nuclear staining was very low. Nuclei appear dark in the middle of the more highly-stained cytoplasm, and confocal imaging also shows negligible nuclear staining. The relative amount of immunofluorescent GAPDH in the nucleus and cytoplasm was quantified by determining the optical density in the nucleus and adjacent cytoplasm; the higher the optical density, the lower the amount of Texas Red-labeled GAPDH. For each experimental group, the nucleus/cytoplasm ratio was calculated from the means of two nuclear and two cytoplasmic values for each cell. Group mean ratios ranged from 1.6 to 2.2 ($n = 8–26$ cells/group), and all ratios were statistically significantly greater than 1.0 ($p < 0.001$), indicating much higher GAPDH levels in cytoplasm compared with nuclei. Also, cells treated with a nitric oxide (NO) donor, sodium nitroprusside (SNP, 100 $\mu\text{mol/L}$) showed low nuclear GAPDH staining (Fig. 3A). There were no statistically significant differences in the GAPDH signal ratios for (i) astrocytes grown in high compared with low glucose for 3 or 4 days, (ii) among groups of low glucose cultures treated with higher doses of SNP (200 $\mu\text{mol/L}$) for 1, 2, or 4h, or (iii) among groups of low-glucose cultures treated with 200, 400, or 800 $\mu\text{mol/L}$ SNP for 1h (data not shown). By visual inspection, treatment with a second NO donor, spermine-NONOate (200 $\mu\text{mol/L}$) for 1.5 or 4h or LPS (1 $\mu\text{g/mL}$ for 48h) did not cause GAPDH to accumulate in nuclei of low-glucose cultures. In other cell types, SNP (200 $\mu\text{mol/L}$ for 1h) or LPS (1 $\mu\text{g/mL}$ for 24h) lead to S-nitrosylation of GAPDH and nuclear translocation (Hara et al., 2005). Thus, the astrocyte cultures in the present study are very resistant to nuclear translocation of GAPDH when exposed to high glucose, NO donors, or LPS.

Nrf2 binds to the antioxidant response element, thereby regulating expression of genes that respond to oxidative stress (Kensler et al., 2007), and when cultured astrocytes were transferred from low to high glucose medium for 15h, treated with thapsigargin to evoke ER stress, or treated with D-glucosamine to enhance the hexosamine pathway, Nrf2 translocated from the cytoplasm to the nucleus in conjunction with increased expression of GRP78 (Takahashi et al., 2012). In the present study, cAMP-treated, differentiated astrocytes were grown in low or high glucose for 14 days, and low-glucose cultures were treated with thapsigargin for 18h to induce ER stress (Fig. 1), and all three groups exhibited strong nuclear Nrf2 staining (Fig. 3B) that contrasts with the prominent cytoplasmic GAPDH staining (Fig. 3A). Optical densities of the anti-Nrf2 stained nucleus and cytoplasm were measured as described above for GAPDH, and the nucleus/cytoplasm optical density ratios were 0.77 ± 0.10 , 0.68 ± 0.12 , and 0.58 ± 0.12 (mean $\pm$ SD, $n = 25$ or 26 cells/group) for the high, low, and thapsigargin-treated low-glucose cultures, respectively. All ratios were

significantly less than 1.0 ($p < 0.001$, one-sample t-test), indicating that the fluorescent anti-Nrf2 staining was greater in the nucleus, thereby reducing its optical density. Nuclear Nrf2 expression in thapsigargin-treated cultures exceeded that in the high and low glucose cultures ($p < 0.05$, ANOVA, Tukey's multiple comparison test). By visual inspection, similar results were obtained for these three experimental groups in cultures that were not treated with cAMP (data not shown), so the presence or absence of differentiation had a modest, if any, effect on Nrf2 nuclear translocation. The nucleus/cytoplasm optical density ratios for a second batch of astrocytes were 0.71 ± 0.14 , 1.06 ± 0.20 , and 0.92 ± 0.19 (mean $\pm$ SD, $n = 24$ – 26 cells/group) for two-week high glucose, low glucose, or low glucose + thapsigargin, respectively. The ratio for the low-glucose group was not significantly different from 1.0, whereas the other two were different ($p < 0.001$ or 0.05 , respectively). Because the ratio of about 1.0 in the low-glucose cultures contrasted with the first data set and suggested similar nuclear and cytoplasmic Nrf2 levels, the data were re-analyzed. The extent of nuclear labeling of Nrf2 in low-glucose culture was found to be heterogeneous, with only 42% of the cells having higher nuclear expression than in the cytoplasm.

3.5. Expression of UPR proteins in STZ-diabetic rat brain and liver

The vehicle-injected, age-matched control rats progressively gained weight during the subsequent 7 months, whereas STZ-diabetic rats had a transient weight loss, followed by a small gain; between 3 and 5 months the STZ rats weighed ~200 g less than controls (Table 1). The plasma glucose levels of STZ-treated rats were elevated ~3-fold and ranged between 27–33 mmol/L. Assuming a brain:plasma distribution ratio for glucose of 0.2 in diabetic rats (Dienel et al., 1991; Holden et al., 1991), brain glucose levels would be in the range of 5.4–6.6 μ mol/g, which is similar to literature values for diabetic animals (Gandhi et al., 2010). STZ-diabetic rats had lower plasma lactate levels compared to controls (Table 1).

Expression of ATF4 was low in diabetic and control cerebral cortex, and the STZ/control ratio was significantly different from 1.0 only at 4 months (Fig. 4A). p-IRE levels were similar in the control and diabetic rats at 1–2 and 4–5 months, but p-IRE was significantly elevated in STZ rats at 3 months (Fig. 4B). ATF6 expression in cerebral cortex was below the detection limit. GRP78 and GRP94 were assayed in cerebral cortex and the inferior colliculus, a midbrain auditory-processing structure that incurs oxidative stress and acquires impairment of gap junctional communication in 5-month STZ-diabetic rats (Gandhi et al., 2010). GRP78 and GRP94 were not expressed at quantifiable levels in cerebral cortex (Fig. 4C-a) or inferior colliculus (not shown) of either control or STZ-diabetic rats at any time point during the 1–5 month interval. Levels of both GRP78 and GRP94 were lower in 4-month STZ rat liver compared with controls (Fig. 4C-b), as observed in the low compared with high glucose cultures (Fig. 2B). iNOS was not detected in cerebral cortex or inferior colliculus of either the STZ or control rats (not shown).

3.6. GAPDH localization and expression of iNOS and amyloid- β in STZ-diabetic brain

We previously found that GAPDH protein levels were reduced in high-glucose astrocyte cultures, but were unchanged in inferior colliculus and cerebral cortex of 5–6-month diabetic rats (Ball et al., 2011). In the present study, anti-GAPDH gave strong bands of similar intensity for blots of 7-month STZ and control cerebral cortex and inferior colliculus, and nuclear translocation of GAPDH was not detected in STZ-diabetic brain (data not shown). Staining of brain sections with anti-iNOS and anti-KDEL was generally weak throughout the brain, except for higher, local labeling of the meninges and tissue immediately around the aqueduct and ventricles (not shown), similar to staining observed for glial fibrillary acidic protein (Ball et al., 2007). Increased amounts of amyloid- β have been reported in 8-month diabetic rats (Li et al., 2007; Cai et al., 2011), but immunostaining of 7-month STZ-diabetic rats with two different anti-amyloid- β antibodies (Sigma A8326 rabbit anti-

amyloid- β_{1-40} , 1:100) or Signet mouse monoclonal anti- β -amyloid (4G8, 1:600) did not reveal any amyloid- β (not shown).

4. Discussion

The major findings of the present study were (i) the lack of a robust unfolded protein response to experimental diabetes in cultured cerebral cortical astrocytes and STZ-rat brain, (ii) failure of astrocytes to respond to LPS or SNP treatments with increases in iNOS level and nitrite production, (iii) undetectable astrocytic nuclear accumulation of GAPDH after treatment with high glucose, LPS, or SNP, and (iv) modest but greater expression of some ER stress markers in low-compared with high-glucose cultures. These observations were unexpected because preparations of astrocytes and of other cell types or lines used in various laboratories did exhibit these types of responses. Unidentified factors in different culture preparations appear to have a strong influence on cellular responses to environmental stresses.

Cultured cerebral cortical astrocytes grown for up to 3–4 weeks in 25 mmol/L glucose and cerebral cortex and inferior colliculus of STZ-treated rats with diabetes for up to 5–7 months did not substantially upregulate the unfolded protein response, and, therefore, do not appear to experience more than mild, if any, ER stress. Two markers for more severe ER stress, p-eIF2 α (translation blockade) and CHOP (apoptotic pathways), and two markers for nitrosative stress (iNOS induction and nuclear translocation of GAPDH) were not detected in high-glucose astrocytes or STZ-diabetic brain. Lack of significant ER stress is consistent with undetectable levels of p-PERK and protein disulfide isomerase, and relatively low levels of ATF4, ATF6, GRP78, and GRP94 in high-glucose cultured astrocytes or STZ-rat brain. However, there was a modest, transient fall in ATF4 and increase in p-IRE expression in STZ-diabetic rat brain. Lack of a robust ER stress response compared with peripheral organs suggests that factors such as high fat diet, obesity, and species differences may contribute to differences in chaperone protein expression in STZ-diabetic rats compared with mice fed a high-fat diet or ob/ob obese mice (Ozcan et al., 2004; Ozcan et al., 2006). Endogenous protective pathways in our cultured astrocytes and STZ-diabetic brain appear to be sufficient to minimize ER stress, which is upregulated in stressed cultured neurons (Murakami et al., 2007) and in brain in vivo during neurodegenerative diseases (Takahashi and Imai, 2003; Paschen and Mengesdorf, 2005; Matus et al., 2011) and cerebral ischemia (DeGracia and Montie, 2004).

Retinopathy and cataracts are prevalent in diabetic patients, and the effects of diabetes and high glucose on the retina have, therefore, been intensively studied. Differences between the diabetic rat retina and brain and between cultured retinal Müller and capillary endothelial cells and cerebral cortical astrocytes are striking. The absence of a robust unfolded protein response, lack of nuclear translocation of GAPDH, and undetectable induction of iNOS levels in high-glucose cultured astrocytes and STZ-diabetic rat brain in the present study sharply contrast with the robust expression in high glucose-treated Müller cells and retina of STZ-diabetic rats of ER stress markers (Li et al., 2009; Chen et al., 2012; Zhong et al., 2012), iNOS (Kern, 2007), nuclear accumulation of GAPDH (Kusner et al., 2004; Kanwar and Kowluru, 2009; Yego et al., 2009; Yego and Mohr, 2010), and apoptosis (Kusner et al., 2004). Apoptosis is also reported in hippocampal neurons in long-duration diabetic rats (Li et al., 2002; Sima and Li, 2005). The basis for greater sensitivity of the retina to diabetes compared with cerebral cortex and other brain regions remains to be established, but breakdown of the blood-retinal barrier is an early event in diabetic retinopathy (Bradbury and Lightman, 1990; Antonetti et al., 1998; Frey and Antonetti, 2011), enhanced inflammatory responses occur in retina during initial stages of diabetes (Kern, 2007), and glucose levels rise 4–7-fold in diabetic retina (Heath et al., 1975; Heath and Hamlett, 1976;

Schofield and Gould, 1979; Poulsom et al., 1983; Tang et al., 2000; Zhang et al., 2003; Puchowicz et al., 2004) compared with 2–3-fold elevations in diabetic brain (Gandhi et al., 2010). The above studies in the Mohr laboratory show that high glucose-evoked nuclear accumulation of GAPDH in Müller cells occurs within 24 h, and GAPDH translocation requires production of active interleukin-1 β and upregulation of *siah-1* protein to transport GAPDH; nuclear translocation is followed by cell death and its blockade is protective. Retinal capillary endothelial cells are also extremely sensitive to high glucose, and <24h exposure is sufficient to evoke UPR and inflammatory responses (Chen et al., 2012; Zhong et al., 2012). The basis for resistance of cerebral cortical astrocytes to hyperglycemia is not known, but improved understanding of this process may help design therapeutic approaches to treat diabetic retinopathy and pathophysiological changes in other organs vulnerable to diabetes.

S-Nitrosylation of GAPDH causes its nuclear translocation (Hara et al., 2006; Tristan et al., 2011; Shahani and Sawa, 2012), suggesting that increased activity of nitric oxide synthase may be a pre-requisite for the high-glucose-induced shift in GAPDH localization. Induction of iNOS appears to require the presence of inflammatory cytokines in cultured astrocytes (Trajkovic et al., 2001; Kuegler et al., 2012). In the present study, cytokines were not evaluated, but various treatment protocols using LPS or SNP did not enhance iNOS expression or nitrite production, whereas similar procedures did cause robust increases in iNOS and nitrite levels in cultured astrocytes (e.g., (Garcia-Nogales et al., 1999) and cited references). However, astrocytes grown in both low and high glucose did exhibit nuclear localization of Nrf2, consistent with upregulation of protective pathways. These findings contrast the results of the careful, detailed study of Takahashi et al. (2012), who reported Nrf2 nuclear localization and GRP78 expression at 18h after transfer of astrocytes from low to high glucose. The basis for these discordant results is not known, but it is not explained by the absence or presence of cAMP treatment to induce astrocytic differentiation. One possibility is that intermittent hypoglycemia coupled with lactate oxidation may precondition the low-glucose cultures and protect against glucose deprivation-induced ER stress. Thus, basal protective processes may be elevated in our astrocyte cultures, minimizing effects of oxidative-nitrosative stress that progressively increases with time in high-compared with low-glucose cultures (Gandhi et al., 2010).

The low glucose cultures expressed higher levels of p-IRE, ATF6, GRP78, and GRP94 compared with high glucose cultures, presumably due to recurrent hypoglycemia arising from cycling between glycolytic and oxidative metabolism according to the feeding intervals. The astrocytes in the present study are unlikely to experience energy failure (as occurs during aglycemia) due to oxidation of lactate. The ability of astrocytes to utilize lactate for gluconeogenesis (Dringen et al., 1993) compensates for the lack of medium-derived glucose and provides a route to generate glucose-6-phosphate for use in the pentose phosphate shunt and other pathways that branch from the glycolytic pathway. In contrast, glucose deprivation of immortalized rat retinal pericytes and capillary endothelial cells does induce ER stress proteins, whereas hyperglycemia does not (Ikesugi et al., 2006; Makita et al., 2011). Growth in high glucose also reduces oxidative metabolism of glucose and lactate by 50% compared with low-glucose cultures (Abe et al., 2006), and glucose level in the culture medium influences flux through the pentose phosphate shunt pathway (Takahashi et al., 2012). Although caution must be exercised when interpreting data obtained in low glucose cultures as a ‘normal control’ condition, these cultures may be a useful model to evaluate effects on astrocytes of periodic hypoglycemic bouts experienced by diabetic patients.

5. Conclusions

Expression of markers for unfolded protein response to ER stress in high-glucose cultured astrocytes and in STZ-diabetic rat brain was undetectable or low. Exposure of astrocytes to oxidative-nitrosative stress secondary to high glucose levels or to nitric oxide donors is sufficient to inhibit astrocytic gap junctional communication, but these conditions are below the threshold required to evoke a strong unfolded protein response or nuclear translocation of GAPDH. Cultured astrocytes and STZ-rat brain are relatively resistant to deleterious effects of high glucose and diabetes compared with Müller cells and retina. ER stress is unlikely to contribute to impaired gap junctional communication among astrocytes and to the early stages of cognitive and neurological deficits in diabetic animals.

Acknowledgments

We thank Dr. Vladimir Lupashin, Department of Physiology and Biophysics, UAMS, for use of the Odyssey imaging system. This work was supported by National Institutes of Health grant DK081936. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of Diabetes and Digestive and Kidney Diseases or the National Institutes of Health. The funding source had no role in study design; collection, analysis, and interpretation of data; writing of the report; and the decision to submit the article for publication.

Abbreviations used

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 2 α
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 1 α
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 2 α
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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Highlights

- Endoplasmic reticulum stress markers are low in high-glucose astrocyte cultures
- Streptozotocin-diabetic rat brain exhibits modest expression of selected markers
- Diabetic astrocytes and brain do not express selected nitrosative stress markers
- Brain and astrocytes are relatively resistant to diabetes-induced ER stress

A. ATF4**A-a. Effect of thapsigargin and STZ diabetes**

Lysis buffer: 50 mmol/L NaCl

Lysis buffer: 150 mmol/L NaCl

A-b. Relative ATF4 level in 14 day cultures **B. p-IRE****B-a. Effect of thapsigargin and STZ diabetes** **B-b. Effect of time in culture** **C. ATF6****C-a. Effect of thapsigargin and STZ diabetes** **C-b. Effect of time in culture** **Fig. 1. Unfolded protein responses in astrocytes cultured in low or high glucose**

Astrocytes were cultured for 3–28 days in low (5.5 mmol/L) or high (25 mmol/L) glucose, and some cultures were treated with thapsigargin (Thaps) or tunicamycin (Tun) to generate a positive control for ER stress (see Methods); the K-562 extract is a commercially-available positive control. Representative blots illustrate the expression of (A-a) ATF4, (B-a, b) p-IRE, and (C-a, b) ATF6 in cultured astrocytes and cerebral cortex of control (Cont) and STZ-diabetic rats. The initial series of assays of the time course of ATF4 used 50 mmol/L NaCl in the lysis buffer, and a prominent band was detected at ~80 kDa (A-a, arrow, left panel), whereas it was ~55 kDa when the NaCl was increased to 150 mmol/L NaCl (A-a, arrow, right panel). Apparent failure to separate ATF4 from putative binding partners (see

Results) compromised the data from the 3–21 day time course analysis. In three additional independent 2-week culture batches extracted with high salt the relative level of ATF4 did not differ in low and high glucose cultures (A-b). p-IRE levels were low in parallel high and low glucose cultures, and were too low to calculate ratios in the third culture batch; values for three additional independent 14-day cultures are also illustrated at the far right of the abscissa in B-b. The high/low glucose ratios for p-IRE levels tended less than 1.0 at each time point, and when all values were pooled, the ratio was significantly less than 1.0 ($p<0.001$). ATF6 also tended to be less than 1.0 at each time point (C-b), and the pooled mean values were significantly less than 1.0 ($p<0.01$). Values are means and SD ($n=3$ independent batches) or individual values from independent batches.

A. GRP78 and GRP94 (KDEL antibody)

B. Temporal profiles of GRP78 and GRP94 levels

C. Glucose and lactate levels in culture medium

Fig. 2. Expression of chaperone proteins, GRP78 and GRP94, in cultured astrocytes and glucose and lactate levels in the culture medium at intervals after medium change

(A) Representative western blot illustrating expression of 94 kDa GRP94 and the 78 kDa GRP78 in extracts of astrocytes cultured for 3–21 days in low or high glucose or in low glucose 33 for 14 days, followed by tunicamycin (Tun) treatment. (B) Representative temporal profiles of signal intensities for GRP78 (left panel) and GRP94 (right panel) with time in culture. GRP78 and GRP94 were assayed in duplicate for each sample of all independent culture batches ($n = 2, 3, 3, 3$, or 1 at 3, 7, 14, 21, and 28 days, respectively), but the signal intensities were variable and very low, particularly for GRP78 in high glucose cultures, so high-to-low glucose ratios were not calculated. (C) Glucose and lactate

concentrations measured in the medium of astrocytes cultured in low (left panel) or high (right panel) glucose at intervals after medium change. Values are means $\pm$ SD (n = 6).

Fig. 3. Localization of GAPDH and Nrf2 in astrocytes cultured in low or high glucose
(A) Representative images of astrocyte cultures immunostained with anti-GAPDH and Texas Red secondary antibody that resulted in bright white staining of cytoplasm and processes, whereas unstained nuclei appear dark (arrows). Top to bottom: astrocytes grown in the presence of cAMP and amphotericin B in low (5.5 mmol/L) or high (25 mmol/L) glucose for 3 days, high glucose for 4 days (confocal image, magnification = 600 $\times$. red = anti-GAPDH, Texas Red; blue = Hoechst dye to stain nuclei, arrow), or low glucose for 5 days then treated with sodium nitroprusside (SNP, 100 μ mol/L) for 48h. Note predominant cytoplasmic labeling by anti-GAPDH and low nuclear staining in all preparations. (B)

Representative images of astrocyte cultures after immunostaining with anti-Nrf2 (white). Astrocytes were grown in the presence of cAMP and amphotericin B for 14 days in 3 conditions: (i) low glucose (5.5 mmol/L; top), (ii) high glucose (25 mmol/L; middle), and (iii) low glucose plus 18h treatment with thapsigargin (bottom). Note the strong nuclear Nrf2 staining in all groups (arrows) that was highest after thapsigargin treatment. The prominent Nrf2 nuclear staining contrasts with that of GAPDH. Scale bars = 75 μ m (magnification = 400 $\times$).

A. ATF4 **B. p-IRE** **C. GRP78 and GRP94 (KDEL antibody)****C-a. Astrocytes vs. control and STZ-rat cerebral cortex (3 and 5 months)** **C-b. Control and STZ-diabetic rat liver (4 months)**

Fig. 4. Expression of ATF4, p-IRE, and GRP78 and GRP94 in STZ-diabetic rat brain and liver
 Extracts of cerebral cortex from age-matched, vehicle-injected control ($n = 4/\text{group}$) and STZ-diabetic rats ($n = 5/\text{group}$) were assayed for expression of (A) ATF4, (B) p-IRE, and (C-a) GRP78 and GRP94. Values in graphs are means $\pm$ SD. Levels of ATF4 were very low, causing variable ratios. (C-a). GRP78 and GRP94 were barely detectable in samples from two independent batches of 28-day high glucose (Hi-Glc) astrocyte cultures and brain from 3 or 5 month control (3-CC, 5-CC) or STZ-diabetic (3-SC, 5-SC) rats, whereas these chaperone proteins were detected in the tunicamycin-treated astrocytes (Tun) and two batches of 28-day low glucose (Lo-Glc) astrocyte cultures that were positive controls. (C-b) Extracts from control and STZ-diabetic rat liver were assayed at 4 months. Levels of GRP78 in STZ-diabetic rats were 80% lower than control liver ($p < 0.001$, t-test); signal intensities were 0.47 ± 0.32 ($n = 5$) and 2.11 ± 0.55 ($n = 4$), respectively, and 4.8 for tunicamycin-

treated astrocytes. GRP94 in STZ-diabetic liver generated higher signal intensities than GRP78 and levels were reduced by about 50% ($p < 0.01$) compared with control liver; signal intensities were 1.59 ± 0.38 ($n = 5$) and 3.34 ± 0.78 ($n = 4$), respectively, and 4.65 for tunicamycin-treated astrocytes.

Table 1

Physiological characteristics of STZ-diabetic and vehicle-control rats.

Group	1 month	2 months	3 months	4 months	5 months	7 months
Bodyweight (g)						
Control	329 ± 15	433 ± 18	447 ± 37	472 ± 16	471 ± 19	482
STZ-diabetic	339 ± 44	256 ± 30	292 ± 26	299 ± 26	268 ± 36	326 ± 55
Plasma lactate (mmol/L)						
Control	3.2 ± 0.9	2.7 ± 0.7	2.8 ± 0.4	2.3 ± 0.4	2.1 ± 0.4	ND
STZ-diabetic	1.9 ± 0.7	1.4 ± 0.2	2.0 ± 0.2	1.7 ± 0.3	1.4 ± 0.3	ND
Plasma glucose (mmol/L)						
Control	10.6 ± 1.9	9.2 ± 1.1	9.8 ± 0.6	11.9 ± 0.9	8.7 ± 0.4	10.3
STZ-diabetic	32.5 ± 5.3	26.9 ± 3.4	31.2 ± 2.5	29.9 ± 3.4	29.1 ± 4.3	30.4 ± 2.9

Body weights and metabolite levels in mixed blood samples from the tails of age-matched, vehicle-injected control (n = 4, except 7 months, n = 1) and STZ-diabetic (n = 5) rats were determined when brains were sampled for analysis of the unfolded protein response at the indicated intervals after the STZ or vehicle injection. Data and tissue samples for the 5-month rats (n = 3/group) were from our previous study (Gandhi et al., 2010). ND denotes not determined.