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Astroglial pentose phosphate pathway rates in response to high-glucose environments

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ABSTRACT

ROS (reactive oxygen species) play an essential role in the pathophysiology of diabetes, stroke and neurodegenerative disorders. Hyperglycaemia associated with diabetes enhances ROS production and causes oxidative stress in vascular endothelial cells, but adverse effects of either acute or chronic high-glucose environments on brain parenchymal cells remain unclear. The PPP (pentose phosphate pathway) and GSH participate in a major defence mechanism against ROS in brain, and we explored the role and regulation of the astroglial PPP in response to acute and chronic high-glucose environments. PPP activity was measured in cultured neurons and astroglia by determining the difference in rate of $^{14}CO_2$ production from [1-^{14}C]glucose and [6-^{14}C]glucose. ROS production, mainly H_2O_2, and GSH were also assessed. Acutely elevated glucose concentrations in the culture media increased PPP activity and GSH level in astroglia, decreasing ROS production. Chronically elevated glucose environments also induced PPP activation. Immunohistochemical analyses revealed that chronic high-glucose environments induced ER (endoplasmic reticulum) stress (presumably through increased hexosamine biosynthetic pathway flux). Nuclear translocation of Nrf2 (nuclear factor-erythroid 2 p45 subunit-related factor 2), which regulates G6PDH (glyceraldehyde-6-phosphate dehydrogenase) by enhancing transcription, was also observed in association with BiP (immunoglobulin heavy-chain-binding protein) expression. Acute and chronic high-glucose environments activated the PPP in astroglia, preventing ROS elevation. Therefore a rapid decrease in glucose level seems to enhance ROS toxicity, perhaps contributing to neural damage when insulin levels given to diabetic patients are not properly calibrated and plasma glucose levels are not adequately maintained. These findings may also explain the lack of evidence for clinical benefits from strict glycaemic control during the acute phase of stroke.

Key words: astrocyte, diabetes mellitus, ER (endoplasmic reticulum) stress, glucose metabolism, Kelch-like enoyl-CoA hydratase-associated protein 1 (Keap1)/nuclear factor-erythroid 2 p45 subunit-related factor 2 (Nrf2), pentose phosphate pathway (PPP).

INTRODUCTION

Brain function is exclusively dependent on the oxidative metabolism of glucose (Clarke and Sokoloff, 1999; Dienel, 2009). Even though the theoretical ratio of the CMR_{oxy} (cerebral metabolic rate of oxygen) and the CMR_{glc} (cerebral metabolic rate of glucose) for the complete oxidation of glucose is 6, the values measured in the human brain during a non-activated, steady state are always lower than 6 (5.0–5.5), indicating that more glucose is consumed than oxidized. This observation has been interpreted as reflecting glucose utilization for the synthesis of neurotransmitters, cellular structural components and release of small amounts of lactate from brain (Clarke and Sokoloff, 1999; Dienel, 2009). When activated, the local CMR_{glc} increases in distinct regions of the brain and transient increases in lactate production (Prichard et al., 1991) associated with the decrease in the CMR_{oxy}/CMR_{glc} ratio are observed (Fox and Raichle, 1986; Fox et al., 1988; Madsen et al., 1999), leading to the hypothesis that activated brain utilizes glucose to produce ATP through

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Abbreviations: 6-AN, 6-aminonicotinamide; [^{14}C]deoxyglucose, 2-deoxy-D-[1-^{14}C]glucose; AGE, advanced glycation end-product; Ara-C, cytosine arabinoside; BiP, immunoglobulin heavy-chain-binding protein; CMR_{glc}, cerebral metabolic rate of glucose; CMR_{oxy}, cerebral metabolic rate of oxygen; DAPI, 4′,6-diamino-2-phenylindole; DBSS, Dulbecco's balanced salt solution; DMEM, Dulbecco's modified Eagle's medium; ER, endoplasmic reticulum; G6PDH, glyceraldehyde-6-phosphate dehydrogenase; H_2DCFDA, 2′,7′-dichlorodihydrofluorescein diacetate; Keap1, Kelch-like enoyl-CoA hydratase-associated protein 1; MCB, monochlorobimane; Nrf2, nuclear factor-erythroid 2 p45 subunit-related factor 2; PERK, double-stranded-RNA-dependent protein kinase-like endoplasmic reticulum kinase; PFA, paraformaldehyde; PLL, poly-L-lysine; PPP, pentose phosphate pathway; ROS, reactive oxygen species; sulforaphane, 1-isothiocyanato-(4*R*,*S*)-(methylsulfinyl)butane.

glycolysis, rather than oxidative phosphorylation (Fox and Raichle, 1986; Fox et al., 1988). Another pathway that contributes to increased glucose utilization without oxygen consumption is the PPP (pentose phosphate pathway), which involves oxidative decarboxylation of carbon one of glucose to generate NADPH for management of oxidative stress; for every six glucose molecules entering the PPP, one glucose equivalent is released as CO_2.

Astroglia play a pivotal role in glucose metabolism for energy production in the brain (Dienel and Hertz, 2005; Hertz et al., 2007). At least one-half or more of the total glucose utilization in the brain has been ascribed to astroglia (Itoh et al., 2004; Nehlig et al., 2004; Hyder et al., 2006). Moreover, a long-lasting debate exists regarding the ANLSH (astrocyte-neuron lactate shuttle hypothesis) (Pellerin and Magistretti, 1994; Magistretti et al., 1999; Chih et al., 2001; Bouzier-Sore et al., 2003; Chih and Roberts, 2003; Pellerin and Magistretti, 2003; Dienel and Cruz, 2004; Hertz, 2004; Hertz et al., 2007; Pellerin et al., 2007; Jolivet et al., 2010), which proposes that the sites of glucose consumption and lactate production in the activated brain are located in astrocytes, irrespective of the controversy regarding the site of the production and neuronal utilization of lactate (Bak et al., 2009; Contreras and Satrustegui, 2009; DiNuzzo et al., 2009; Mangia et al., 2009). However, the benefit, if it exists, of such compartmentation of glucose metabolism in neurons and astroglia remains uncertain. These facts and controversy suggest that the excess utilization of glucose compared with oxygen in the brain as a whole may be clinically relevant to the disease pathology, and that if the glycolytic metabolism of glucose predominates in astrocytes, these cells may play important roles through their glucose metabolism in the pathophysiology of metabolic disorders of the brain, such as diabetic encephalopathy. Although a possible role of glycolysis as a survival pathway rather than an energy production pathway has been postulated (Bolaños et al., 2010), glycolysis in astroglia has not been extensively studied as in cultured neurons (Bolaños et al., 2010).

As for neuronal energy production dependent on mitochondrial oxidative metabolism of glucose, ROS (reactive oxygen species) derived from the mitochondria in neural cells may play a harmful role in stroke (Mehta et al., 2007) and aging of the brain (Kregel and Zhang, 2007), as well as neurodegenerative disorders (Jomova et al., 2010). Hyperglycaemia is well known to enhance ROS production in vascular endothelial cells, resulting in vascular diseases (Brownlee, 2001, 2005). Despite the fact that diabetes mellitus is a risk factor for dementia of either the vascular or Alzheimer's type (Biessels et al., 2006; Xu et al., 2009), whether the hyperglycaemic state causes direct neuronal cell damage remains controversial (Sommerfield et al., 2004; Cox et al., 2005; McCall, 2005; Ryan, 2006; Manschot et al., 2007; Sima, 2010). Even though a hyperglycaemic state is harmful to parenchymal cells (i.e. neurons and astroglia), the deleterious effects of a hyperglycaemic state do not seem to be as devastating as they are to vascular endothelial cells. In a clinical setting, an elevation in the blood glucose concentration is often observed during the acute phase of stroke in patients, regardless of pre-existing diabetes mellitus, and is associated with a poor prognosis (Capes et al., 2001). Despite the potential harmful effects of hyperglycaemia on brain parenchymal cells in animal studies (Macdougall and Muir, 2011), the beneficial effects of lowering the blood glucose level during the acute phase of stroke have not been confirmed by clinical trials (Quinn and Lees, 2009; Kruyt et al., 2010; McCormick et al., 2010). Although hyperglycaemia is now generally accepted to exacerbate ischaemic damage, a recent survey of clinical studies suggests that mild hyperglycaemia is actually beneficial in cases with lacunar infarction (Uyttenboogaart et al., 2007). These facts may indicate that an appropriate glucose content in the brain is necessary to protect brain cells against ischaemic cell damage.

The PPP branches from the glycolytic pathway of glucose at glucose 6-phosphate, and the activity of the rate-limiting enzyme of the PPP, G6PDH (glyceraldehyde-6-phosphate dehydrogenase), is regulated by NADPH, peroxynitrite and other factors, as well as by transcriptional mechanisms (Dringen et al., 2007; Wamelink et al., 2008). The PPP is generally considered to be a minor pathway of glucose metabolism in resting brain, but it can be markedly up-regulated under various conditions (e.g. Appel and Parrot, 1970) to generate NADPH, which in turn increases GSH to detoxify ROS in concert with glutathione peroxidase (Dringen et al., 2007). 'Resting' PPP rate and the magnitude of PPP increase during activation are known to be higher in astrocytes than in neurons, although they are not negligible in neurons (Ben-Yoseph et al., 1996a, 1996b; Delgado-Esteban et al., 2000; García-Nogales et al., 2003; Vaughn and Deshmukh, 2008; Herrero-Mendez et al., 2009). Gandhi et al. (2010) reported increased oxidative stress in cultured astrocytes grown in high glucose and in the brain of diabetic rats, and we therefore hypothesized that acute and chronic hyperglycaemic states activate the PPP in astroglia to protect the brain. In the present study, we explored the mechanisms that are activated to regulate PPP under acute and chronic hyperglycaemic conditions, focusing on ER (endoplasmic reticulum) stress and the Keap1 (Kelch-like enoyl-CoA hydratase-associated protein 1)/Nrf2 (nuclear factor-erythroid 2 p45 subunit-related factor 2) system, which is a master regulator of phase-2 detoxifying enzymes such as G6PDH (Thimmulappa et al., 2002).

MATERIALS AND METHODS

Animals

Timed-pregnant Sprague–Dawley rats were purchased from Japan SLC, Inc. All animal procedures were performed in accordance with The Animal Experimentation Guidelines of

Keio University School of Medicine and were approved by the Committee on Animal Care and Use, Keio University.

Chemicals

Chemicals and materials were obtained from the following sources: [^{14}C]deoxyglucose (2-deoxy-D-[1-^{14}C]glucose; specific activity, 2.13 GBq/mmol), Insta-Fluor Plus and hyamine hydroxide 10-X were obtained from PerkinElmer Life Sciences; D-[1-^{14}C]glucose (specific activity, 2.035 GBq/mmol) and D-[6-^{14}C]glucose (specific activity, 2.035 GBq/mmol) were obtained from American Radiolabeled Chemicals; normal goat serum was obtained Jackson ImmunoResearch; mouse anti-KDEL monoclonal antibody was obtained from Stressgen; rabbit anti-Nrf2 (H-300) polyclonal antibody, rhodamine-conjugated goat anti-rabbit IgG antibody and FITC-conjugated goat anti-mouse IgG antibody were obtained from Santa Cruz Biochemistry; mouse anti-N^{ε}-(carboxyethyl)lysine antibody was obtained from Cosmo Bio Co. Ltd; DMEM (Dulbecco's modified Eagle's medium) with or without glucose, penicillin and streptomycin were obtained from Life Technologies; defined fetal bovine serum was obtained from HyClone Laboratories; H_2DCFDA (2′,7′-dichlorodihydrofluorescein diacetate) and MCB (monochlorobimane) were obtained from Molecular Probes; and all other chemicals were obtained from Sigma.

Preparation of cells

Primary astroglial cultures were prepared from the cerebral cortex of rat pups 24–48 h after birth (Takahashi et al., 1995). The dissociated cells from the frontoparietal cortices (2.5×10^5 cells/ml) were plated (15 ml/flask) in uncoated 75 cm^2 culture flasks (Sumitomo Bakelite) and cultured in a glucose-containing medium (final concentration, 12 mmol/l of D-glucose) comprised of DMEM with 10% (v/v) fetal bovine serum, penicillin (100 units/ml) and streptomycin (100 μg/ml) at 37°C in humidified air containing 7% CO_2 (day 0). The culture medium was changed every 2 days until the cultures reached confluence. On day 11, the adherent cells were treated with trypsin/EDTA solution, suspended in fresh high (12 mmol/l)-glucose medium, and placed in uncoated 12- and 24-well culture plates (0.8 and 0.4 ml/well respectively) (Nalge Nunc) or in 25 cm^2 culture flasks (5 ml/flask) (Nalge Nunc). For immunohistochemical staining, the cells were plated (2 ml/dish) on 35 mm glass-bottomed dishes (Matsunami Glass Industry) precoated with PLL (poly-L-lysine). Some cells were grown on glass-bottomed 24-well culture plates (EZView culture plate LB) for fluorimetric assay. From the day after subculturing, the cells were cultured in DMEM, fetal bovine serum, penicillin and streptomycin and a final D-glucose concentration of 5, 12 or 23 mmol/l for 10 days. The culture medium was changed twice a week, and the cells were used once they reached confluence (on day 21 *in vitro*).

The primary neuronal and mixed neuronal–astroglial cultures were prepared from the cortex and striatum of fetal rats on embryonic day 16, as described previously (Takahashi et al., 1995). The mechanically dissociated cells were placed in 12- and 24-well culture plates (0.8 and 0.4 ml/well, respectively) or 25 cm^2 culture flasks (5 ml/flask) coated with PLL (5 μg/ml). For the neuronal cultures, viable cells (1.5×10^6 cells per ml) that excluded Trypan Blue were placed in the cultures and Ara-C (cytosine arabinoside; 10 μmol/l) was added 72 h later to induce the mitotic arrest of the astroglia. For mixed neuronal–astroglial cultures, 1.0×10^6 cells per ml were placed in PLL-coated plates and no Ara-C was added. The cells were cultured in a glucose medium (final concentration, 12 mmol/l D-glucose) at 37°C in humidified air containing 7% CO_2. *In vitro* assays were performed using cultures that were 7 or 8 days old. The nutrient medium remained untouched until the experiments were initiated. When neurons were grown on astroglial culture, the astroglial cultures were prepared as described above; on culture day 21, the neuronal cells were seeded on the astroglial cell layer and Ara-C was added 72 h later. The cells were used for the assay 7 days after the neurons had been seeded.

Experimental protocol

To assess the effects of acutely changing concentrations of D-glucose (2, 10, 20 mmol/l) on glucose metabolism, ROS production and immunohistochemistry, the nutrient medium (12 mmol/l) was removed and the cells were washed twice with PBS without Ca^{2+} and Mg^{2+} containing no glucose and the cells were incubated with DBSS (Dulbecco's balanced salt solution) containing 110 mmol/l NaCl, 5.4 mmol/l KCl, 1.8 mmol/l $CaCl_2$, 0.8 mmol/l $MgSO_4$, 0.9 mmol/l NaH_2PO_4 and 44 mmol/l $NaHCO_3$ in addition to 2, 10 or 20 mmol/l of D-glucose supplemented with 18, 10 or 0 mmol/l L-glucose respectively. The L-glucose was added to the culture media so the medium osmolarity was the same for each experimental group. To assess the chronic effects of high-glucose environments, astroglial cells were cultured with DMEM containing 5 (low-glucose medium) or 23 (high-glucose medium) mmol/l D-glucose for 10 days, as described above. The glucose concentrations during the assay procedure were 2, 10 or 20 mmol/l. Specific assay conditions are indicated in the Figure legends for each experiment.

When astroglial cells were exposed to sulforaphane [1-isothiocyanato-(4*R*,*S*)-(methylsulfinyl)butane; 10 μmol/l] – an Nrf2 activator, thapsigargin (1 μmol/l) – an ER stress inducer or D-glucosamine (1–10 mmol/l) – a precursor of *N*-acetylglucosamine generated through the hexosamine biosynthetic pathway (another minor pathway of glucose metabolism), the nutrient medium (5 mmol/l D-glucose) was replaced with fresh medium (5 mmol/l D-glucose) containing drugs of the indicated concentrations or appropriate vehicles for 15 h. To evaluate the effect of different glucose environments, immunohistochemically, astroglial cells that had been cultured in a low-glucose medium (5 mmol/l) for

10 days, were exposed to DBSS containing 2, 10 or 20 mmol/l D-glucose for 15 h. To assess the effects of long-term exposure to different glucose environments on ROS production, cells were cultured with DMEM containing 5 or 23 mmol/l D-glucose for 10 days before ROS measurement.

Assay for [^{14}C]deoxyglucose phosphorylation

The rates of glucose phosphorylation in astroglia were evaluated using a modification (Takahashi et al., 1995; Abe et al., 2006a) of the [^{14}C]deoxyglucose method (Sokoloff et al., 1977). The culture medium of a 12-well plate was replaced with 0.4 ml of DBSS containing 2, 10 and 20 mmol/l D-glucose supplemented with 18, 10 and 0 mmol/l L-glucose respectively. To keep the specific activity of [^{14}C]deoxyglucose constant as the concentration of D-glucose was increased, 12.5, 62.5 or 125 μl/ml of [^{14}C]deoxyglucose (original concentration: 3.7 MBq/ml) was added to each assay solution and incubation was continued for 60 min at 37°C in 7% CO_2. At the end of the incubation period, the DBSS was replaced with a fresh reaction mixture lacking [^{14}C]deoxyglucose and incubation was continued at 37°C in 7% CO_2 for 5 min to allow the efflux of residual [^{14}C]deoxyglucose from the cells (Takahashi et al., 1995; Abe et al., 2006a). This procedure allows the evaluation of glucose phosphorylation activity instead of simple glucose uptake activity. The cell carpets were washed quickly three times with ice-cold PBS and digested in 1.0 ml of 0.1 mM NaOH at room temperature. The cell digests were then assayed for protein content using the BCA (bicinchoninic acid) method (Smith et al., 1985), and the ^{14}C count was measured using a liquid scintillation counter (Tri-Carb 3100TR; PerkinElmer Life Sciences). The rates of glucose phosphorylation (pmol of glucose/μg of protein per 60 min) based on the conversion from [^{14}C]deoxyglucose phosphorylation over 60 min were then calculated from the specific activity of [^{14}C]deoxyglucose (i.e. mBq of [^{14}C]deoxyglucose/mmol of glucose) in the reaction mixture.

Measurement of the rate of D-[1-^{14}C]glucose and D-[6-^{14}C]glucose oxidation to $^{14}CO_2$

The rate of [^{14}C]glucose oxidation to $^{14}CO_2$ was measured using a modification of a previously described method (Abe et al., 2006a). After cells cultured in 25 cm^2 culture flasks had been washed twice with PBS containing no glucose, the assay solutions were added and the flasks were capped with rubber stoppers containing a centre well and incubated at 37°C for 60 min. The $^{14}CO_2$ produced was trapped by a cotton ball placed in the centre well containing 100 μl of hyamine hydroxide 10-X. The reactions were terminated by the injection of 250 μl of 60% $HClO_4$ through the rubber stopper, and the flasks were kept at 4°C overnight to trap the $^{14}CO_2$. The centre wells were then transferred to 20 ml glass scintillation counter vials, and 500 μl of ethanol and 10 ml of Insta-Fluor Plus were added. The ^{14}C contents of the vials were evaluated using a liquid scintillation counter. The assay solutions consisted of 2.5 ml of DBSS containing 2, 10 or 20 mmol/l D-glucose supplemented with 18, 10 or 0 mmol/l L-glucose respectively; these solutions were labelled by adding 1.0, 5.0 or 20.0 μl/ml of D-[1-^{14}C]glucose or D-[6-^{14}C]glucose (original concentrations: 3.7 MBq/ml) to keep the specific activities constant.

Waniewski and Martin (2004) reported that substantial ^{14}C counts were obtained from a flask without cells. Therefore the ^{14}C counts obtained from a flask without cells in which the reaction had been stopped at 60 min were regarded as the background values and were subtracted in our studies. The cell carpets remaining in the incubation flasks after the removal of the reaction mixtures were then digested with 5 ml of 0.1 M NaOH, and their protein contents were determined. The rates of total glucose oxidation (pmol of glucose/μg of protein per 60 min) based on the conversion from [^{14}C]glucose into $^{14}CO_2$ over 60 min were measured.

Measurement of PPP activity in cultured astroglia and neurons

PPP activity was measured using a modification of the method described by Hothersall et al. (1979). Briefly, cells were incubated with glucose with tracer doses of [1-^{14}C]glucose or [6-^{14}C]glucose for 60 min and PPP rate calculated as the difference between the $^{14}CO_2$ derived from [1-^{14}C]glucose (metabolized by both PPP and the tricarboxylic acid cycle) and that derived from [6-^{14}C]glucose (metabolized by only the tricarboxylic acid cycle) was thought to be an indicator of the PPP activity. To confirm the validity of this tracer assay, 15 h prior to and during the PPP assay, 6-AN (6-aminonicotinamide; 500 μmol/l), a competitive inhibitor of G6PDH, was added to the nutrient medium and assay solution, and the PPP activity was measured. PPP is known to be active in proliferating cells because PPP yields ribose 5-phosphate for nucleotide biosynthesis, leading to DNA and RNA synthesis. To evaluate the effects of the proliferation of astroglia on PPP activity, Ara-C (10 μmol/l) was added to the astroglial culture medium 48 h prior to the assay to arrest cell division and the PPP activity was evaluated.

Measurement of ROS production

The production of ROS, mainly H_2O_2, in cells was assessed using H_2DCFDA (Gomes et al., 2005) and semi-quantitative fluorimetric measurements. Just prior to the assay, the nutrient medium was removed and the cells were washed twice with PBS without glucose. Then, DBSS containing 10–30 μmol/l H_2DCFDA dissolved in DMSO (final volume of DMSO: 0.1%) supplemented with 2 mmol/l glucose were added and the cells were incubated at 37°C in humidified air with 7% CO_2 for 30 min. After loading the H_2DCFDA for 30 min, the cells were washed twice again with PBS without glucose and DBSS containing 2, 10 or 20 mmol/l D-glucose

with 18, 10 or 0 mmol/l L-glucose added respectively. The cells were further incubated for 60 min, and the fluorescence level indicating intracellular ROS production was measured at 0 and 60 min using a fluorescence microplate reader (Infinite 200 PRO; Tecan Japan, Kanagawa, Japan; or Cytofluor 4000J; Applied Biosystems Japan) with λ_{ex} at 485 nm and λ_{em} at 530 nm. As the fluorescence signals increased linearly for up to 60 min (data not shown), the results were expressed as the percentage increase in the fluorescence signal at 60 min compared with that at 0 min.

Measurement of GSH content using a fluorimetric method

The intracellular content of GSH in astroglia was assessed using MCB which forms an adduct with GSH via an enzymatic reaction catalysed by glutathione transferases (Chatterjee et al., 1999). Cells were incubated with MCB (50 μmol/l) for 30 min. The intracellular formation of the GSH–MCB adduct was assessed using a microplate reader with λ_{ex} at 360 nm and λ_{em} at 460 nm. The results were expressed as the percentage increase in the fluorescence signal over 60 min.

Immunohistochemistry

BiP (immunoglobulin heavy-chain-binding protein) expression, an ER stress indicator, or the translocation of Nrf2 from the cytosol to the nucleus was assessed using immunohistochemistry. For detection of AGEs (advanced glycation end-products), immunostaining of N^{ε}-(carboxyethyl)lysine was performed. Astroglial cells that were grown on a glass-bottomed, 35 mm dish were fixed with 4% (w/v) PFA (paraformaldehyde) for 10 min on ice. Then the cells were permebilized with 0.5% Triton X-100 for 10 min at room temperature, followed by post-fixation with 4% PFA for 5 min on ice. For immunostaining, the cells were washed twice with PBS containing $MgCl_2$ and $CaCl_2$, and non-specific IgG binding sites were blocked by incubating the cells in PBS containing 3% BSA and 3% normal goat serum for 30 min at room temperature. Cells were incubated with the primary antibodies (mouse anti-KDEL monoclonal antibody, 1:200; anti-Nrf2 polyclonal antibody, 1:100 or mouse anti-N^{ε}-(carboxyethyl)lysine antibody, 1:100 for 2 h at room temperature). Then, the cells were incubated with the secondary antibodies (rhodamine-conjugated goat anti-rabbit IgG antibody or FITC-conjugated goat anti-mouse IgG antibody) with DAPI (4′,6-diamino-2-phenylindole) for nuclear staining for 1 h. The cells were examined using a laser confocal microscopy system (Leica TCS SP5; Leica Microsystems).

Statistical analyses

Statistical comparisons among the values obtained for each group were performed using grouped *t* tests or a one-way ANOVA followed by the Dunnett test for multiple comparisons. A $P<0.05$ was considered statistically significant.

RESULTS

Effects of acute and chronic exposure to elevated extracellular glucose concentrations on glucose phosphorylation in cultured astroglia

Normal brain glucose concentration is approximately 20–25% that in arterial plasma and it generally falls within the range of 2–3 and 5–7 μmol/g in normal and diabetic rat brain respectively (Gandhi et al., 2010). When astrocytes were grown in culture medium containing 5, 12 or 23 mmol/l glucose, then assayed for rates of glucose utilization with tracer amounts of [^{14}C]deoxyglucose in the reaction mixture containing 2 mmol/l glucose, there were no differences among the cultures in the rates of glucose phosphorylation (Figure 1A). In contrast, when astrocytes were grown in 12 mmol/l glucose and assayed for glucose utilization rate in 2, 10 or 20 mmol/l glucose, the rate of glucose phosphorylation fell by approximately 20% when the glucose level in the assay mixture was much lower than that of the culture medium and it increased by approximately 15% when glucose level in the assay medium was elevated; glucose phosphorylation rate varied linearly with respect to D-glucose concentration in the assay mixture (Figure 1B).

Higher rates of glucose utilization when assay glucose levels exceeded 2 mmol/l were unexpected findings because the K_m of brain hexokinase for glucose is approximately 0.05 mmol/l (Grossbard and Schimke, 1966), and as long as the enzyme is saturated (>1 mmol/l glucose) the reaction rate would not change. Differences in the rate of [^{14}C]deoxyglucose phosphorylation can, however, arise from changes in the value of the lumped constant of the deoxyglucose method, the factor that accounts for differences in transport and phosphorylation of deoxyglucose and glucose (Sokoloff et al., 1977). The value of the lumped constant increases sharply in the hypoglycaemic range, but it is relatively stable during hyperglycaemia (Orzi et al., 1988; Schuier et al., 1990), decreasing by ~20% as brain glucose level rises 2.5-fold from 2 to 5 μmol/g (Dienel et al., 1991). Values for the lumped constant are not available for higher brain glucose levels and have not been determined in different brain cell types, so changes in its value were not were not taken into account in the present study. However, correction of measured [^{14}C]deoxyglucose phosphorylation rates for changes in the value of the lumped constant would be expected to modestly increase the calculated glucose phosphorylation rates (Figure 1B) determined in 10 and 20 mmol/l D-glucose compared with that in 2 mmol/l D-glucose and to slightly increase the slope of the regression line. Also, these effects are not due to osmotic changes in the culture medium, because the total D-/L-glucose concentrations were kept constant by adding L-glucose, a biologically inactive form of glucose, when the D-glucose concentration was reduced.

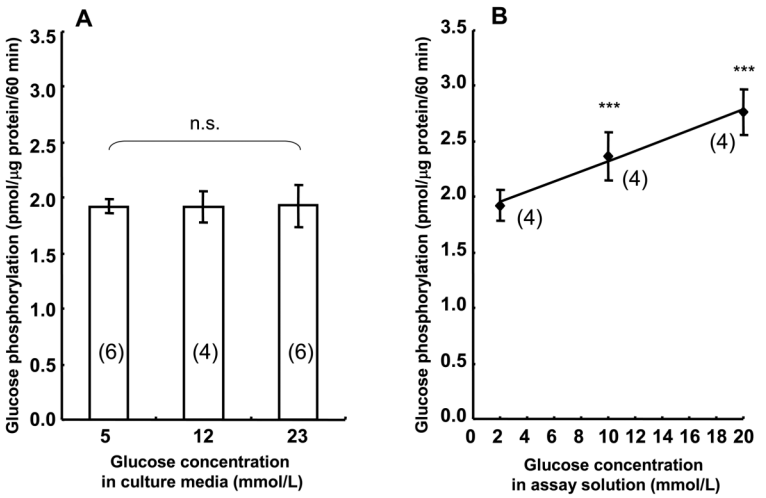

Figure 1 **Effects of D-glucose concentration on rate of [^{14}C]deoxyglucose phosphorylation in astroglia grown (A) or assayed (B) in different concentrations of D-glucose**

Glucose utilization rates were assayed with [^{14}C]deoxyglucose in astrocytes chronically exposed to different glucose levels for 10 days, then assayed in 2 mmol/l D-glucose (**A**) or cultured in 12 mmol/l D-glucose for 10 days, then assayed at the indicated D-glucose concentration (**B**). Increasing the glucose concentration in the assay medium elicited statistically significant increases in the rates of glucose phosphorylation; the continuous line is the linear regression: $y=0.0463x+1.8575$ ($r^2=0.991$) (**B**). Note that rates of glucose phosphorylation were calculated based on the specific activity of [^{14}C]deoxyglucose (i.e. mBq of [^{14}C]deoxyglucose/mmol of glucose) in the assay medium and do not include a factor for the lumped constant, so the actual rates of glucose phosphorylation would be higher than the calculated values (see the Results section). Values are means ± S.D. for four to six wells (numbers in parentheses); n.s., not significant (ANOVA); ***$P<0.001$ (ANOVA followed by Dunnett test for multiple comparisons).

Note that the rates of glucose phosphorylation in Figure 1 were calculated using the specific activities of [^{14}C]deoxyglucose in the assay mixtures and do not include the lumped constant in the denominator. Actual glucose utilization rates are therefore higher than the calculated rates because the lumped constant accounts for the slightly higher transport rate (approximately 1.4-fold) and the 3-fold lower rate of phosphorylation rate for deoxyglucose compared with glucose; for *in vivo* experiments, the lumped constant is 0.48 (Sokoloff et al., 1977), indicating that two glucose molecules are phosphorylated for each deoxyglucose molecule phosphorylated. The rates in Figure 1 are therefore not directly comparable with rates of $^{14}CO_2$ production from [1- and 6-^{14}C]glucose oxidation (see below). To summarize, changes in glucose utilization rate with acute change in glucose level from the culture medium to the assay mixture arise mainly from unidentified effects that are unrelated to the maximal rate of hexokinase or the lumped constant and probably involve regulation of glucose metabolism at downstream sites.

PPP rate in cultured astroglia exceeds that in cultured neurons and is influenced by glucose level in the PPP assay mixture and culture medium

PPP rates were determined separately in cultured astroglia and neurons as the difference (Figure 2C) between the rates of $^{14}CO_2$ production from oxidation of [1-^{14}C]glucose (Figure 2A) and [6-^{14}C]glucose (Figure 2B). Glucose oxidation via the tricarboxylic acid cycle (i.e. $^{14}CO_2$ production from [6-^{14}C]glucose) was higher in neurons compared with astrocytes (Figures 2A and 2B), but the PPP activity in astroglia was approximately 4–5 times higher than in neurons (Figure 2C), consistent with previous reports (e.g. Ben-Yoseph et al., 1996a, 1996b; García-Nogales et al., 2003).

Decarboxylation of [1-^{14}C]glucose by astroglia was almost completely eliminated by pre-treatment with 6-AN, and PPP activity was reduced to approximately 3% of control by 6-AN (Table 1). As the PPP activity of the astroglia was unaffected by Ara-C-induced mitotic arrest (Table 1), the higher PPP activity measured in the cultured astroglia, compared with that in neurons (Figure 2), cannot be ascribed to proliferative activity.

When astrocytes were grown in 12 mmol/l glucose and PPP activity assayed at different glucose concentrations, the rate of decarboxylation of [1-^{14}C]glucose was lower or higher when the glucose level in the assay medium was less than or exceeded that in the culture medium respectively (Figure 3A). On the other hand, the decarboxylation rate of [6-^{14}C]glucose was quite low and not altered by differences in glucose level the assay compared with growth media (Figure 3B). Both [1-^{14}C]glucose decarboxylation and PPP rates were linearly related to glucose level in the assay medium (Figures 3A–3C). To summarize, acute changes in glucose concentration in the assay medium compared with the culture medium cause parallel changes in glucose utilization rate (Figure 1B) and

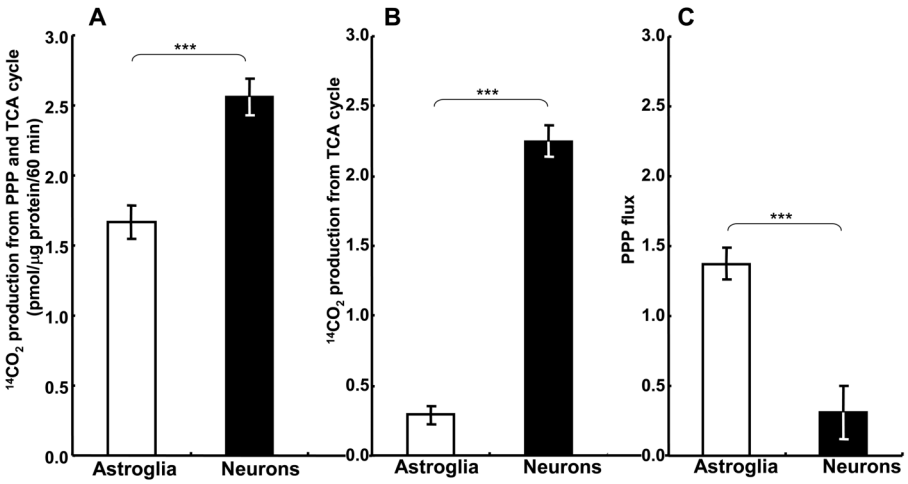

Figure 2 **Rates of $^{14}CO_2$ production from oxidation of [1-^{14}C]glucose (A) and [6-^{14}C]glucose (B), and rate of the PPP, calculated as the difference in $^{14}CO_2$ production rates ([1-^{14}C]glucose minus that from [6-^{14}C]glucose) (C) in astroglia (white bars) and neurons (black bars)**

Cells were cultured in 12 mmol/l glucose and the assay was performed in 2 mmol/l glucose. Values are means ± S.D. for quadruplicate flasks; ***P<0.001 (grouped t test).

Table 1 **Modulation of PPP rates**

Rates of $^{14}CO_2$ production from oxidation of [1-^{14}C]glucose and [6-^{14}C]glucose were used to calculate the rate of the PPP as the difference in $^{14}CO_2$ production rates ([1-^{14}C]glucose minus that from [6-^{14}C]glucose) in astroglia treated with vehicle, 500 μmol/l 6-AN (6-aminonicotinamide) for 15 h or 10 μmol/l Ara-C for 48 h. Cells that had been cultured with 5 mmol/l D-glucose were assayed in the presence of 2 mmol/l D-glucose. Values are means ± S.D. for quadruplicate flasks. *P<0.001; †not significant (grouped t test).

Treatment	[1-^{14}C]Glucose oxidation (pmol/μg of protein per 60 min)	[6-^{14}C]Glucose oxidation (pmol/μg of protein per 60 min)	PPP rate (pmol/μg of protein per 60 min)
Control for 6-AN	1.27 ± 0.11	0.08 ± 0.05	1.19 ± 0.13
6-AN treated for 15 h	0.18 ± 0.05*	0.14 ± 0.06†	0.04 ± 0.06*
Control for Ara-C	1.89 ± 0.14	0.59 ± 0.03	1.31 ± 0.12
Ara-C treated for 48 h	1.79 ± 0.18†	0.62 ± 0.09†	1.33 ± 0.22†

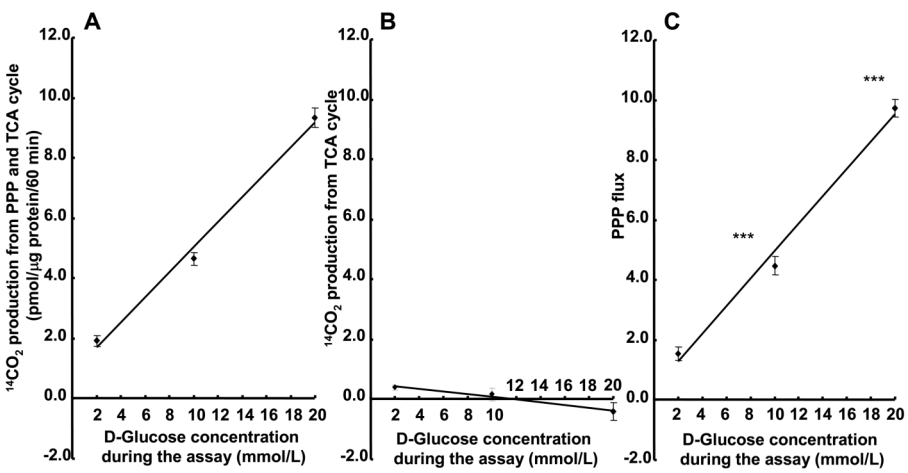

Figure 3 **Effects of acutely changing concentrations of D-glucose on rate of $^{14}CO_2$ production from oxidation of [1-^{14}C]glucose (A) and [6-^{14}C]glucose (B), and PPP rate (C)**

Continuous lines are regression lines; that for the PPP in (**C**) is y=0.4584x+0.3574 (r^2=0.9907). Astroglia were cultured in 12 mmol/l glucose before the assay of PPP activity at different glucose concentrations as indicated. Values are means ± S.D. for quadruplicate flasks; ***P<0.001 (ANOVA followed by Dunnett test for multiple comparisons).

PPP rate (Figure 3C). This finding has important implications for interpretation of results of metabolic assays in cultured cells because cells are often grown in higher glucose levels than those used for *in vitro* assays using radiolabelled substrates (i.e. to maximize the specific activity and sensitivity of the assay), and large changes in glucose level in the assay system can cause artifactual changes in glucose utilization and pathway fluxes.

Cultured endothelial cells (Brownlee, 2001, 2005), astrocytes (Gandhi et al., 2010 and references cited therein) and other cell types grown in media containing high glucose for various periods of time ranging from 2–3 days to 3–4 weeks are used as models for experimental diabetes mellitus. PPP activity was, therefore compared in astroglia grown in media containing 5 or 23 mmol/l glucose, and then assayed in reaction mixtures containing 2 or 20 mmol/l glucose. When astroglia were cultured in a high-glucose environment (23 mmol/l glucose) for 10 days, PPP rates were statistically significantly enhanced compared with cells grown in 5 mmol/l glucose when measured in assay medium containing either 2 or 20 mmol/l glucose (Figure 4). The predominant effect increased rate of decarboxylation of [1-^{14}C]glucose, with small, if any, changes in rates of decarboxylation of [6-^{14}C]glucose with chronic or acute differences in glucose level (Figure 4). PPP rates were much higher when assayed in 20 mmol/l compared with 2 mmol/l glucose, regardless of the growth medium glucose level, and growth in high glucose and assay in high glucose evoked the highest PPP activity (Figure 4). Acute changes in glucose concentration had a greater effect of PPP activity than prolonged exposure to high glucose (Figure 4). These findings demonstrate that acute upward or downward shifts in glucose concentration cause PPP activity to fall or rise (Figures 3 and 4) in parallel with changes glucose phosphorylation rate (Figure 1).

Influence of acute or chronic exposure of astrocytes and neurons to different glucose levels on ROS generation and on level of GSH in astrocytes

Prolonged exposure of cultured astrocytes to 25 mmol/l glucose increases ROS production (Gandhi et al., 2010), and the present study evaluated ROS production in astrocytes and neurons during acute changes in glucose concentration. Astroglia and neurons were grown in 12 mmol/l glucose for 10 or 7–8 days respectively and then assayed for ROS production in different glucose concentrations. In astroglia, ROS production decreased by approximately 85% when acutely exposed to 20 mmol/l glucose compared with 2 mmol/l glucose (Figure 5A). In contrast, ROS production was higher in neurons than in astroglia and it increased as the D-glucose concentration was elevated (100, 115 and 145% at 2, 10 and 20 mmol/l glucose respectively) (Figure 5B). Thus, neurons are more sensitive to acute variations in glucose level than astroglia, consistent with greater neuronal sensitivity to other treatments that evoke oxidative stress (e.g. Ben-Yoseph et al., 1996a, 1996b; García-Nogales et al., 2003). Neither mixed cultures of neurons and astroglia (Figure 6A) nor neurons grown on an astroglial cell layer (Figure 6B) showed statistically significant alterations in ROS production with increased glucose level, despite the fact that neurons alone responded to acutely increasing concentrations of D-glucose by elevated ROS production. These results suggest that astrocytes convey protection to neurons against acute high-glucose-induced oxidative stress.

PPP activation by acute exposure to 20 mmol/l glucose occurred in astrocytes grown in either 5 or 23 mmol/l, with a higher PPP response in the cells grown in 23 mmol/l glucose medium (Figure 4). In spite of the large differences in PPP activities in the low- and high-glucose cultures, the ROS levels assayed in the same glucose level were similar in the

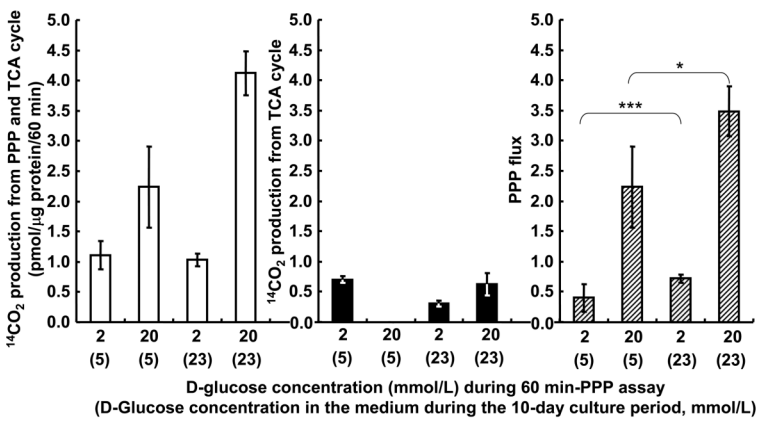

Figure 4 **Effects of chronic exposure to low (5 mmol/l) or high (23 mmol/l) D-glucose environments on PPP activities in response to acutely changing the D-glucose concentrations (2 or 20 mmol/l) during the assay**
Rate of $^{14}CO_2$ production from oxidation of [1-^{14}C]glucose (white bars) and [6-^{14}C]glucose (black bars), and PPP rate (hatched bars) in astroglia measured at the indicated glucose concentrations during the assay. The glucose concentration in the culture medium is indicated in parentheses under the glucose level in the assay medium. Values are means ± S.D. for quadruplicate flasks; *P<0.05 ***P<0.001 versus vehicle (grouped t test).

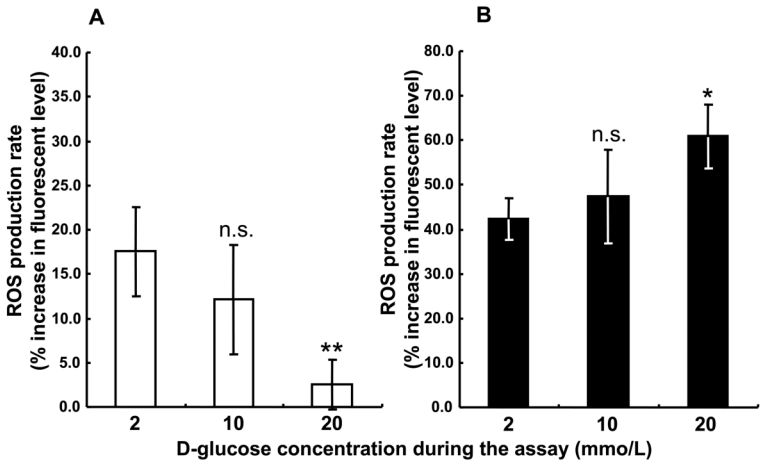

Figure 5 **Effect of acutely changing concentrations of D-glucose on rates of ROS production determined by H_2DCFDA fluorescence in astroglia (A) and neurons (B)**

Values are means ± S.D. for quadruplicate wells; n.s., not significant; *$P<0.05$, **$P<0.01$ versus 2 mmol/l glucose (ANOVA followed by Dunnett test for multiple comparisons). Both astroglia and neurons were cultured in 12 mmol/l glucose for 10 and 7 days respectively and the assay was performed in 2, 10 and 20 mmol/l glucose.

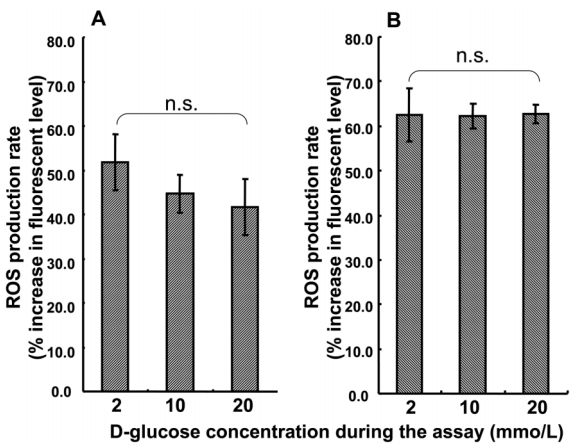

Figure 6 **Effect of acutely changing concentrations of D-glucose on rate of ROS production determined by H_2DCFDA fluorescence in mixed astroglia–neuron cultures (A) and neurons grown on an astroglial cell layer (B)**

Values are the means ± S.D. of quadruplicate wells; n.s., not significant versus 2 mmol/l glucose (ANOVA followed by Dunnett test for multiple comparisons). Cells were cultured in 12 mmol/l glucose for 10 days and the assay was performed in 2, 10 and 20 mmol/l glucose.

astrocytes cultured in either 5 or 23 mmol/l glucose (Table 2). In other words, ROS production in astroglia cultured in a high-glucose medium did not show a significant reduction compared with that in astroglia cultured in a low-glucose medium, even though PPP activity was higher. These findings are unexpected, because astroglia that had been cultured in high-glucose environment (23 mmol/l) shows higher PPP rates at 2 and 20 mmol/l (Figure 4). We speculated that increases in AGE in astroglia that had been cultured in high-glucose environment (23 mmol/l) might have opposing

Table 2 **Modulation of ROS formation**

Effects of chronic exposure to low (5 mmol/l) or high (23 mmol/l) D-glucose environments on ROS production was determined by H_2DCFDA fluorescence in response to acutely changing D-glucose concentrations (2, 10 or 20 mmol/l) during the assay. Astroglia were cultured in 5 or 23 mmol/L D-glucose, and ROS measured at the indicated concentrations of D-glucose during the assay. Results are means ± S.D. for four wells; *not significant (grouped *t* test).

Conditions D-Glucose concentrations during the assay (mmol/l)...	2	10	20
Cultured with 5 mmol/l D-glucose for 10 days	84.7 ± 5.3	66.9 ± 10.5	68.1 ± 6.7
Cultured with 23 mmol/l D-glucose for 10 days	85.7 ± 7 7*	69.6 ± 12.2*	68.6 ± 13.8*

Table 3 **Modulation of the level of GSH**

Effect of acutely changing concentrations of D-glucose on the level of GSH in astroglia treated with or without 500 μmol/l L-buthionine sulfoximine, a γ-glutamylcysteine synthase inhibitor, for 48 h. Astroglia were cultured in 12 mmol/l glucose before the assay of GSH content that was performed at different glucose concentrations.Means ± S.D. for four wells; *not significant; †$P<0.01$ (ANOVA followed by Dunnett test for multiple comparisons); ‡$P<0.01$ (grouped t test).

Conditions D-Glucose concentrations during the assay (mmol/l)...	2	10	20
L-Buthionine sulfoximine (without)	23.7 ± 1.9	29.5 ± 2.4*	32.8 ± 5.2†
L-Buthionine sulfoximine (with)	3.3 ± 1.5‡	6.5 ± 2.5*‡	8.5 ± 1.7†‡

influence on ROS production. Therefore we also performed immunohistochemical analysis of N^{ε}-(carboxyethyl)lysine, one of major AGE, in astroglia (see Figure 11 and the Discussion section).

Acutely altering glucose concentrations also modulated the GSH content in astroglia. There was a tendency for GSH to be lower when assayed in 2 mmol/l glucose compared with 10 mmol/l and GSH was significantly higher when assayed in 20 mmol/l glucose compared with 2 mmol/l (Table 3). L-buthionine sulfoximine reduces the synthesis of glutathione by inhibiting γ-glutamylcysteine synthase, and incubation of astroglia with this drug for 48 h markedly reduced GSH content, but the glucose concentration dependence of GSH level was still evident (Table 3). These findings suggest that the higher PPP activity regenerates GSH and can maintain higher GSH levels even when oxidative stress is higher. Taken together, all of the above findings indicate that chronically elevated glucose levels enhance PPP activity, and acute increases in glucose level stimulate both glucose utilization and PPP activity to minimize ROS production and sustain levels of GSH in astroglia that serve a protective role for neurons exposed to acute glucose-evoked oxidative stress.

Chronic high-glucose environments induce ER stress, trigger nuclear translocation of Nrf2 and up-regulate PPP activity

PPP activity in astroglia is known to be regulated by the Nrf2-mediated transcription of G6PDH (Thimmulappa et al., 2002), so the underlying mechanisms that regulate PPP activity in the presence of chronic high-glucose environments were explored. First, the effect of sulforaphane, an Nrf2 activator, on PPP activity in astroglia was examined to confirm that the Nrf2-mediated regulatory system of the PPP was active in our cultured astroglia. PPP activity doubled in astroglia after 15 h

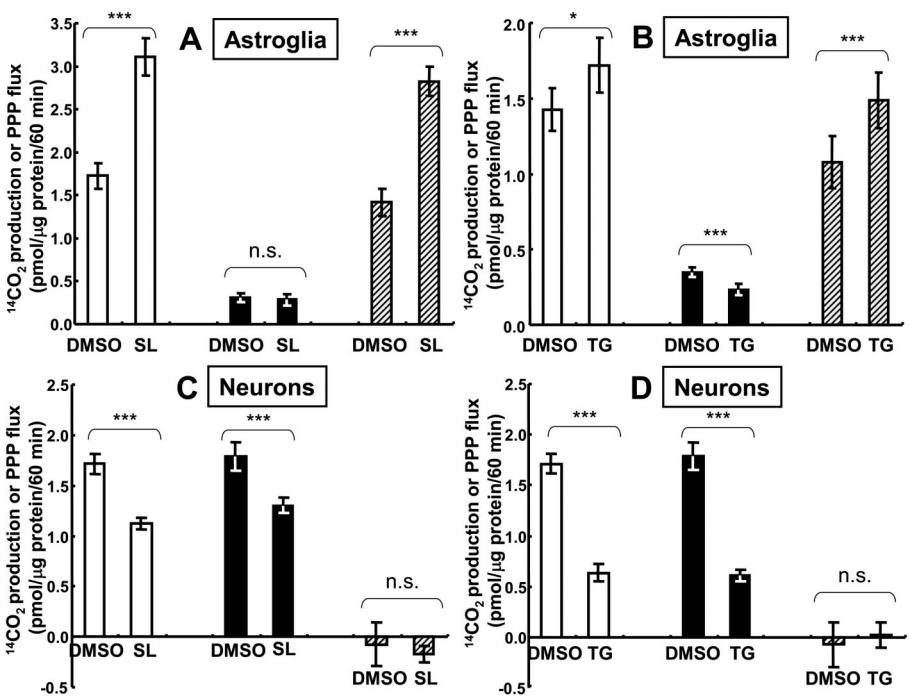

Figure 7 **Effects of 10 μmol/l SL (sulforaphane) or 1 μmol/l TG (thapsigargin) compared with DMSO vehicle on rate of $^{14}CO_2$ production from oxidation of [1-^{14}C]glucose (white bars) and [6-^{14}C]glucose (black bars), and PPP rate (hatched bars) in astroglia (A, B) and neurons (C, D)**

Values are means ± S.D. for quadruplicate flasks; n.s., not significant; *$P<0.05$; ***$P<0.001$ versus vehicle (grouped t test). Cells were cultured in a low-glucose (5 mmol/l) medium and the assay was performed in 2 mmol/l glucose.

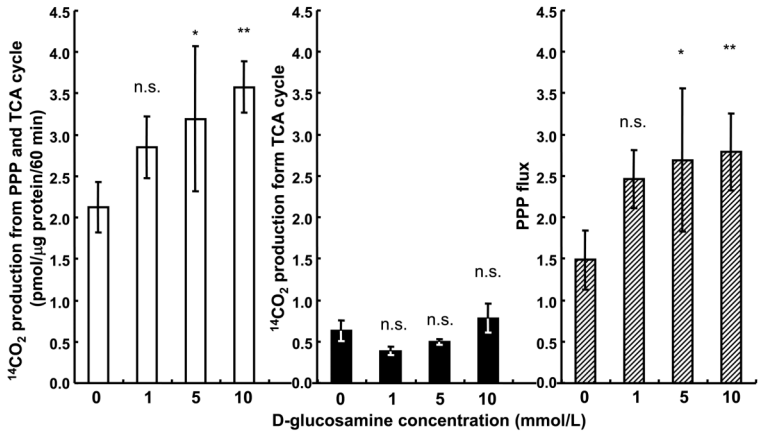

Figure 8 **Effects of D-glucosamine on rate of $^{14}CO_2$ production from oxidation of [1-^{14}C]glucose (white bars) and [6-^{14}C] glucose (black bars), and PPP rate (hatched bars) in astroglia**

Values are means ± S.D. of quadruplicate flasks; n.s., not significant; *P<0.05; **P<0.01 versus control treated with vehicle (ANOVA followed by Dunnett test for multiple comparisons). Cells were cultured in a low-glucose (5 mmol/l) medium, then the medium was replaced with fresh medium containing the indicated concentration of D-glucosamine for 15 h, and the assay was performed in 2 mmol/l glucose.

of treatment with 10 μmol/l sulforaphane (Figure 7A). Secondly, we examined whether ER stress induces PPP activation. Nrf2 is reportedly a direct substrate of PERK (double-stranded-RNA-dependent protein kinase-like ER kinase), an ER stress transducer protein kinase, and the phosphorylation of Nrf2 facilitates its dissociation from the adaptor protein Keap1 and its translocation to the nucleus (Surh et al., 2008; Vargas and Johnson, 2009). Thapsigargin (1 μmol/l), a well-established inducer of ER stress (Rasheva and Domingos, 2009), elicited PPP activation in astroglia after 15 h (Figure 7B). Sulforaphane increased decarboxylation of [1-^{14}C]glucose without altering glucose oxidation in the tricarboxylic acid cycle (Figure 7A), whereas thapsigargin increased decarboxylation of [1-^{14}C]glucose and reduced that of [6-^{14}C]glucose (Figure 7B). Importantly, these two pathways were not activated in neuronal cells. Sulforaphane (Figure 7C) and thapsigargin (Figure 7D) reduced rates of oxidation of [1-^{14}C]glucose and [6-^{14}C]glucose and also suppressed PPP activities in neurons without affecting the morphology of the neurons after 15 h of exposure to these drugs (data not shown), indicating that the lack of PPP activation was not due to gross neuronal cell damage.

It is well known that high-glucose environments can induce *N*-acetylglucosamine synthesis through an increased flux into the hexosamine biosynthetic pathway, which leads to abnormal glycosylation of proteins and triggers ER stress (Brownlee, 2001, 2004). This pathway involves conversion of the glycolytic intermediate fructose 6-phosphate into glucosamine 6-phosphate, followed by its acetylation to generate *N*-acetylglucosamine 6-phosphate that subsequently enters the glycosylation pathways. We hypothesized that the hexosamine pathway plays an important role in the regulation of the PPP in astroglia and measured the PPP activities after incubation for 15 h with D-glucosamine (1–10 mmol/l), a precursor of the hexosamine pathway. D-glucosamine did, indeed, induce PPP activation in a dose-dependent manner (Figure 8).

First, we examined the effects of sulforaphane, thapsigargin and D-glucosamine on expression of BiP, an ER stress marker, and nuclear translocation of Nrf2 were assessed in cultured astroglia. Fifteen hours of exposure to low-glucose (5 mmol/l) medium plus sulforaphane caused the translocation of Nrf2 from the cytosol to the nucleus without inducing BiP expression (compare Figure 9B with control Figure 9A). Both thapsigargin (Figure 9C) and D-glucosamine (Figure 9D) induced BiP expression and the nuclear translocation of Nrf2.

Next, the influence of low- and high-glucose environments on expression of BiP and nuclear translocation of Nrf2 were assessed in cultured astroglia. Elevation of D-glucose concentration from 5 to 10 or 20 mmol/l for 15 h induced Nrf2 translocation to the nucleus and increased BiP expression at 10 and 20 mmol/l of glucose (Figure 10), indicating that high glucose stimulates the pathway that enhances G6PDH mRNA transcription and causes ER stress, presumably via the hexosamine pathway. Because treatment with sulforaphane and thapsigargin (Figures 7A and 7B) and D-glucosamine (Figure 8) increased the PPP activity in astroglia, ROS production rates were also assessed after 15 h of incubation with sulforaphane, thapsigargin and D-glucosamine (Table 4). All treatments reduced ROS production compared with control conditions and enhanced the PPP activity (Table 4). Together, these findings indicate that ROS production in astroglia is regulated by Keap1/Nrf2-dependent PPP activation. We also observed that long-term culture (6 weeks) in high-glucose medium (23 mmol/l) enhanced immunostaining of AGEs as compared with short-term culture (3 weeks) in high-glucose medium (23 mmol/l) or in low-glucose medium (5 mmol/l) for both 3 and 6 weeks (Figure 11).

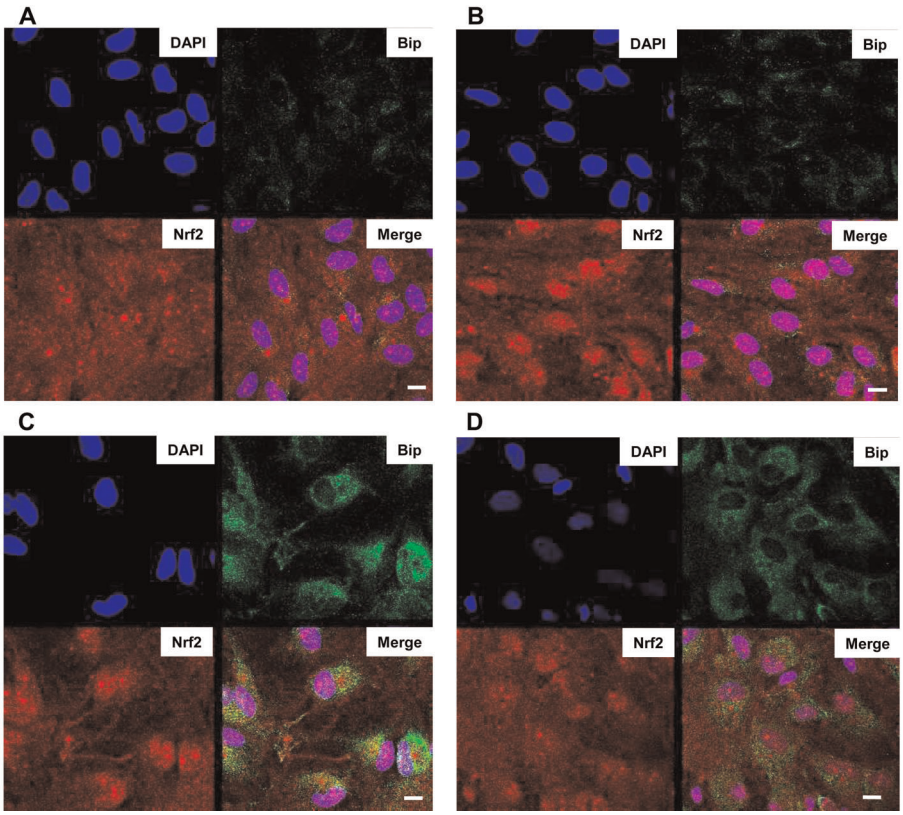

Figure 9 **Immunohistochemical staining of astroglia grown in a low-glucose (5 mmol/l) medium, then treated for 15 h with DMSO vehicle (control, A), 10 μmol/l sulforaphane (B), 1 μmol/l thapsigargin (C) and 10 mmol/l D-glucosamine (D)**
DAPI for nuclear staining (blue), BiP (green), Nrf2 (red) and overlay in each panel. The scale bars indicate 10 μm. Nrf2 in astroglia treated with vehicle shows a diffuse distribution throughout the cell, including the nucleus and cytosol (**A**). Sulforaphane facilitates the nuclear translocation of Nrf2 without inducing BiP expression in astroglia (**B**). Thapsigargin (**C**) and D-glucosamine (**D**) induced BiP expression with a reticular distribution and increased Nrf2 nuclear localization, indicating that ER stress is associated with Nrf2 translocation to the nucleus.

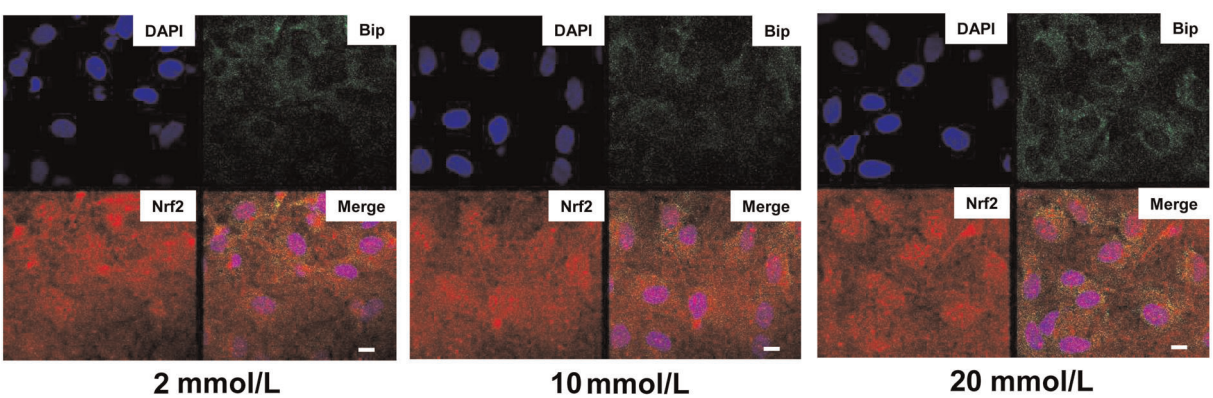

Figure 10 **Effects of high-glucose environments on ER stress and Nrf2 localization in astroglia**
Cells were cultured in a low-glucose medium containing 5 mmol/l D-glucose for 10 days before exposure to DBSS containing 2, 10 or 20 mmol/l D-glucose for 15 h. DAPI for nuclear staining (blue), BiP (green), Nrf2 (red) and overlay in astroglia. Then 15 h exposure to high-glucose environments (10 or 20 mmol/l) induced ER stress, as indicated by BiP expression and the nuclear translocation of Nrf2, while exposure to the normal brain glucose level (2 mmol/l) environment did not. Scale bars indicate 10 μm.

Table 4 **Modulation of ROS formation**

ROS production was assayed after treatment with vehicle, sulforaphane, thapsigargin, or D-glucosamine for 15 h. Cells that had been cultured with 5 mmol/l D-glucose were assayed in the presence of 2 mmol/l D-glucose.

Treatment	Rate of ROS production (%)	PPP rate (pmol/μg of protein per 60 min)
Sulforaphane (μmol/l)		
0	63.0 ± 5.5	1.42[c]
10	57.5 ± 4.1†	2.83*** [c]
Thapsigargin (μmol/l)		
0	72.6 ± 3.6	1.08 [d]
1	51.2 ± 3.0‡	1.49*** [d]
D-Glucosamine (mmol/l)		
0	76.4 ± 3.9	1.49 [e]
1	74.5 ± 1.1[a]	2.47[ns e]
5	65.4 ± 3.8[b]	2.70* [e]
10	64.3 ± 3.9[b]	2.79** [e]

Means ± S.D. for six wells; †$P<0.05$; ‡$P<0.01$ (grouped t test).
[a]Not significant.
[b]$P<0.01$ (ANOVA followed by Dunnett test for multiple comparisons).
[c]Data from Figure 7(A).
[d]Data from Figure 7(B).
[e]Data from Figure 8 (see respective Figure legends for definitions of statistical significance).

DISCUSSION

The major findings of the present study are that both acute and chronic exposure to high-glucose environments enhanced the PPP activities, thereby controlling glucose-evoked ROS production in cultured rat astroglia through different and co-operative mechanisms. In contrast, ROS production rises in cultured neurons under high-glucose conditions, and the PPP in neurons does not respond to conditions that up-regulate PPP in astrocytes. However, neurons are protected against oxidative stress evoked by acute increases in glucose level by co-culture with astrocytes. These actions of astroglia indicate a potential protective role against oxidative stress in the brain under the hyperglycaemic conditions associated with diabetes mellitus. As shown in Figures 6(A) and 6(B), these roles of astroglia seem to be independent of cultivation period (1 week versus 4 weeks), developmental stage *in vivo* (embryonic day

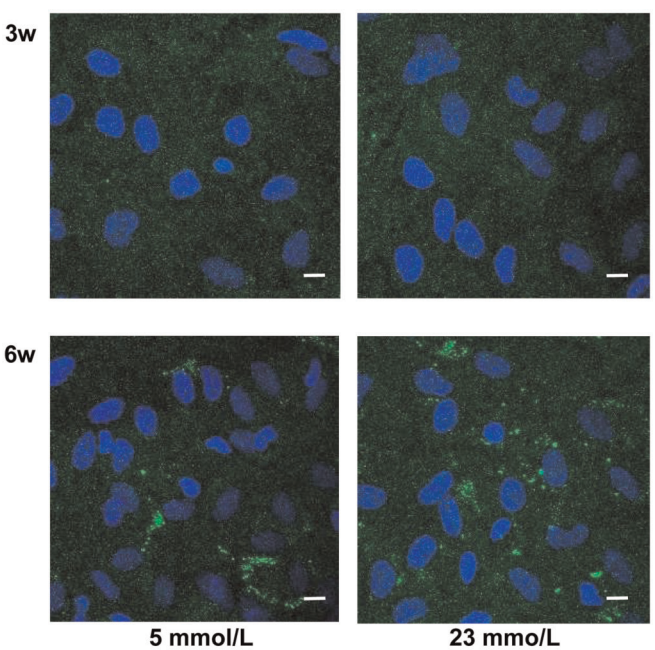

Figure 11 **Effects of high-glucose environments on N^{ε}-(carboxyethyl)lysine, a major AGE (green), and DAPI for nuclear staining (blue), in astroglia**

Cells were cultured in a low-glucose (5 mmol/l) or high-glucose (23 mmol/l) medium for 3 weeks (3 w, upper panels) or 6 weeks (6w, lower panels). Scale bars indicate 10 μm.

16 versus newborn) or regions of the brain (striatum versus cerebral cortex) in accordance with our previous report that showed similar metabolic properties of glucose in cultured astroglia prepared from striata of fetal rats on embryonic day 16 (Abe et al., 2006b). Notably, if astroglia are acutely shifted to a lower glucose concentration compared with that in the assay medium, the PPP rate is down-regulated and ROS levels increase, suggesting increased vulnerability of brain cells to oxidative stress during transient episodes of reduced glucose level that are normally adequate to support brain function (i.e. 2 mmol/l) or transitory hypoglycaemia that is commonly experienced by diabetic patients.

In addition to their clinically relevant implications, the results of this study reveal important issues related to *in vitro* metabolic assays. The sensitivity of changes in astroglial glucose metabolism, pathway fluxes and ROS generation in response to extracellular glucose concentrations needs to be taken into account when designing experimental procedures for *in vitro* metabolic assays and assays that may be sensitive to oxidative stress in cultured cells. Also, the consequences of (i) cyclic changes of glucose concentrations as a result of periodic change of culture media (i.e. feeding astroglial cells), (ii) culture of astrocytes and neurons in media containing extremely high glucose concentrations (>15–20 mmol/l) that greatly exceed glucose levels in diabetic rat brain (5–7 μmol/g; Gandhi et al., 2010) and (iii) the progressive depletion of glucose from culture medium that is not changed in order to minimize neuronal damage or death that can arise from medium change need to be taken into account when interpreting results.

Acutely increasing concentrations of D-glucose increased the glycolytic flux into the PPP, enhancing decarboxylation at carbon 1 of D-glucose, as evidenced by the increase in [1-^{14}C]glucose-derived $^{14}CO_2$ production. A slight but definite decrease in [6-^{14}C]glucose-derived $^{14}CO_2$ production indicated that astroglial mitochondrial oxidative phosphorylation in the tricarboxylic acid cycle was suppressed (Figure 3B), whereas glucose utilization increased, as assessed by the rates of [^{14}C]deoxyglucose phosphorylation (Figure 1B). The overall rate of brain glucose utilization is regulated in an integrated, complex manner by various regulatory metabolites at many steps, including at the initial step in glycolytic pathway, the phosphorylation of glucose to glucose 6-phosphate by hexokinase, as well as at many downstream sites, especially, phosphofructokinase (the major regulatory enzyme), pyruvate kinase, PDH (pyruvate dehydrogenase) and tricarboxylic acid cycle dehydrogenases. PPP fluxes are governed by NADP availability due to consumption of NADPH to regenerate GSH from GSSG as the cell manages oxidative stress (Dringen et al., 2007). Nitric oxide and peroxynitrite also strongly activate the PPP (García-Nogales et al., 2003), as do various neurotransmitters that are metabolized by monoamine oxidase and generate H_2O_2 (e.g. Appel and Parrot, 1970; Ben-Yoseph et al., 1996a, 1996b and cited references). Brain hexokinase has a low K_m value (approximately 0.05 mmol/l), compared with the glucokinase with a high K_m that is typically found in the liver and is not inhibited by glucose-6-phosphate, as is hexokinase (Grossbard and Schimke, 1966). As a result, hexokinase is saturated at glucose concentrations above 1 mmol/l, consistent with the fact that CMR_{glc} does not increase in response to acute hyperglycaemia *in vivo* (Orzi et al., 1988; Schuier et al., 1990). There is evidence for the existence of glucokinase activity in the brain (Roncero et al., 2000, 2004, 2009; Alvarez et al., 2002) and its presence in cultured astrocytes is not ruled out.

Our previous report, showing that chronic high-glucose environments reduced astroglial oxidative metabolism of glucose (Abe et al., 2006a) might have some relevance to results of the present study. Chronic high-glucose environments also increased PPP activities in astroglia, while glucose utilization (as assessed using [^{14}C]deoxyglucose phosphorylation measured at 2 mmol/l glucose) was not altered (Figure 1A). Importantly, the PPP activities in astroglia that had been cultured at 23 mmol/l glucose for 10 days were higher than those in astroglia cultured at 5 mmol/l for 10 days measured at either 2 or 20 mmol/l glucose (Figure 4). These results indicate that chronic high-glucose environments enhance PPP activities preserving the dependence of PPP activities on glucose concentrations. Therefore some additional mechanisms may act to elevate the PPP activities at any concentration of D-glucose. To explore the underlying mechanisms, we focused on the transcriptional regulation of G6PDH, a rate-liming enzyme of the PPP.

The G6PDH gene is known to possess an antioxidant response element and the Keap1/Nrf2 system stimulates G6PDH transcription under stressed conditions and this response is quite rapid (i.e. within 24 h) (Thimmulappa et al., 2002). Nrf2 is a transcriptional factor that is kept in the cytosol under unstressed conditions, forming a complex with Keap1, an anchor protein bound to the cytoskeleton. The Keap1–Nrf2 complex is constantly degraded by the proteasome system; thus, the transcriptional activity of Nrf2 is suppressed under normal physiological environments (Surh et al., 2008; Vargas and Johnson, 2009). At least two different triggers are known to facilitate Nrf2 translocation from the cytosol to the nucleus to bind the antioxidant response element. One trigger is a modification of thiol residues in the Keap1–protein, typically as a result of an increase in ROS (Surh et al., 2008; Vargas and Johnson, 2009). Sulforaphane, a natural isothionate found in broccoli sprouts, is a potent activator of Nrf2 (Danilov et al., 2009; Cheung and Kong, 2010; Guerrero-Beltrán et al., 2010) and may activate PPP via this mechanism. Another mechanism is the phosphorylation of serine residues in the Nrf2 protein (Surh et al., 2008; Vargas and Johnson, 2009). Several kinases reportedly phosphorylate Nrf2 at Ser^{40} (Bloom, 2003; Surh et al., 2008; Vargas and Johnson, 2009). Cullinan et al. (2003) reported that Nrf2 is a direct substrate of PERK, a kinase that acts as a transducer of ER stress (Rasheva and Domingos, 2009). Thus, ER stress could trigger the Keap1/Nrf2 system-dependent transcriptional regulation of type 2 detoxifying enzymes

(Cullinan, 2004; Cullinan and Diehl, 2006; Xu et al., 2010). First, we confirmed that sulforaphane enhances PPP activity and Nrf2 translocation without BiP expression, a well-known ER stress marker (Rasheva and Domingos, 2009) in astroglia. Secondly, we found that thapsigargin, an ER stress inducer (Rasheva and Domingos, 2009) did, indeed, enhance BiP expression, as well as the nuclear translocation of Nrf2, in astroglia in association with the enhancement of PPP activity in astroglia.

We found that the PPP activity in astroglia is approximately 4–5 times higher than that in neurons. The PPP is well known to be active in proliferating cells because the PPP is necessary for DNA synthesis (Wamelink et al., 2008). However, the proliferating activity in astroglia was found to be of no relevance to the high activity of the PPP observed in cultured astroglia because mitotic arrest by Ara-C did not affect the PPP activity. In addition to the measurement of the basal PPP activity in neurons, we attempted to activate the neuronal PPP activity using sulforaphane or thapsigargin. Neither of these agents, however, altered the neuronal PPP activities. The enhanced activity of PPP in astroglia did, indeed, decrease the rates of ROS production in both astroglial and neuronal cells when cultured together. Although the measured ROS production in the fluorimetric system is the sum of the generation and elimination of the ROS, the observed decreases in the rate of ROS production in the astroglia may reflect enhanced elimination or decreased generation. The present observation that acutely increasing glucose concentrations elicited increases in GSH content and that these increases were further suppressed by the addition of L-buthionine sulfoximine, an inhibitor of glutathione synthesis (Table 3) supports the latter possibility.

In fact, the PPP has been reported to be more active in astroglia than in neurons (e.g. Ben-Yoseph et al., 1996a, 1996b; García-Nogales et al., 2003), as found in the present study. As mitochondrial respiratory chain is an important source of ROS in the brain, astroglial glutathione may play a key role in protecting against neuronal damage caused by ROS. GSH in astroglia is reportedly released into the extracellular space to reduce ROS and is then transferred to neurons for protection. Acute hyperglycaemia increases ROS production in the endothelium (Nishikawa et al,. 2000; Brownlee, 2001, 2005), and the neuronal cultures in our assay also exhibited the enhanced production of ROS under acutely increasing glucose concentrations. In contrast, the ROS production in astroglia was reduced by increases in the glucose concentrations. Interestingly, in mixed cultures of neurons and astroglia, the ROS production remained constant as glucose concentrations were elevated. We speculated that increases in astroglial GSH may reduce neuronal ROS production in our cultured system, as reported by García-Nogales et al. (2003). In fact, Asanuma et al. (2010) demonstrated that zonisamide, which has been used clinically as an anti-epileptic drug, augments astroglial GSH levels and resultant increases in cysteine transfer to dopaminergic neurons confer a novel protective mechanism against neuronal degeneration through quenching ROS and dopamine quinone in Parkinson's disease model *in vivo*.

Finally, we explored the regulatory mechanism of the PPP via the Keap1/Nrf2 system in the presence of chronic hyperglycaemia. We focused on hexosamine biosynthetic pathway in astroglia (Matthews et al.. 2007), because this pathway is known to produce ER stress (Kline et al., 2006; Sage et al., 2010) under hyperglycaemic conditions (Özcan et al., 2004). In the present study, an intermediate metabolite of the hexosamine pathway, D-glucosamine, did indeed trigger both ER stress and Nrf2 translocation to the nucleus, and the PPP activities were enhanced, as evidenced by the results of the [^{14}C]glucose assay. These actions of D-glucosamine were also confirmed by dose-dependent decreases in ROS production in astroglia. In addition, chronic hyperglycaemia induced BiP expression and the nuclear translocation of Nrf2, as evidenced by the immunohistochemical analyses. Taken together, acute and chronic hyperglycaemia enhanced the PPP activity using different but co-operative pathways.

Many clinical observations show that long-term hyperglycaemia causes vascular diseases (Brownlee, 2001, 2005). However, the adverse effects of hyperglycaemia on the brain *per se*, i.e. diabetic encephalopathy, remain controversial (Sommerfield et al., 2004; Cox et al., 2005; McCall, 2005; Ryan, 2006; Manschot et al., 2007; Sima, 2010). One possible reason may be that the brain possesses a potent protective mechanism against high-glucose-induced ROS. The present study provides one possible mechanism by which primarily the astroglia play a pivotal role in neuroprotection against ROS under hyperglycaemic conditions. In addition, these actions of astroglia are dependent on the existence of D-glucose. These results imply that reducing the glucose concentrations too rapidly or by too large an extent may have detrimental effects on the astroglial protective system. In fact, reducing the blood glucose level during the acute phase of stroke does not necessarily have a beneficial effect on stroke mortality and functional recovery. Moreover, the fluctuation of blood glucose levels, rather than sustained hyperglycaemia, is reported to be a more potent risk factor for stroke (Monnier et al., 2006) and dementia, reflecting brain parenchymal cell damage (Fowler and Vasudevan, 2010).

Astroglia always responds to acutely increasing glucose concentrations by enhancing PPP rate irrespective of the culture conditions, but basal PPP rate is higher when cells were cultured in high glucose. For example, astroglia cultured for 10 days in a high-glucose environment (23 mmol/l) exhibited enhanced PPP rate at 2 or 20 mmol/l D-glucose as compared with PPP rate measured at corresponding D-glucose concentration in astroglia that had been cultured in a low-glucose environment (5 mmol/l) (Figure 4). However, the net production of ROS production by astroglia that had been cultured in 5 or 23 mmol/l glucose did not differ at 2, 10 and 20 mmol/l respectively (Table 2). These apparently-conflicting results imply that the enhancement of ROS generation is overcome under high-glucose environments irrespective of

the enhanced activity of ROS elimination. We speculate that a non-enzymatic reaction that produces methylglyoxal from glyceraldehyde 3-phosphate, another minor pathway of glucose metabolism, may be involved. Methylglyoxal generates AGEs that are well-known sources of ROS under a chronic hyperglycaemic state (Dhar et al., 2008; Lo et al., 2010). The increased production of methylglyoxal and resultant increases in ROS production by AGEs in a hyperglycaemic state have been reported in the vascular system (Dhar et al., 2008; Lo et al., 2010). If the adverse effects of high-glucose environments overcome the astroglial intrinsic protective mechanism in the long run, they may result in brain damage. Recent findings that experimental diabetes increases production of ROS–reactive nitrogen species and inhibits astrocytic gap junctional communication in tissue culture and brain slices from streptozotocin-diabetic rats suggest that astroglial dysfunction does, indeed, occur after longer period of disease duration irrespective of intrinsic self-defence mechanisms (Gandhi et al., 2010; Ball et al., 2011). Also, we observed increased immunostaining of N^{ε}-(carboxyethyl)lysine, one of the major AGEs, in astroglia that had been cultured in 23 mmol/l glucose for 6 weeks (Figure 11), supporting the above possibility. Astroglial dysfunction could be involved in the pathogenesis of dementia (Biessels et al., 2006; Xu et al., 2009) or Parkinson's disease (Cereda et al., 2011; Schernhammer et al., 2011; Xu et al., 2011), which have recently been found to be more common in diabetic patients.

In conclusion, astroglia play a neuroprotective role under acute and chronic high-glucose conditions, extending the findings of previous studies related to protective effects of the PPP against oxidative stress induced by neurotransmitters and neuroregulators and their metabolites. Both of these acute and chronic conditions depend on an appropriate glucose content; thus, maintaining glucose concentrations in the proper range is relevant for the astroglial PPP as a neuroprotective mechanism.

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