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Cyanide preconditioning protects brain endothelial and NT2 neuron-like cells against glucotoxicity: Role of mitochondrial reactive oxygen species and HIF-1α

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ABSTRACT

The current study was undertaken to address the role of mitochondrial reactive oxygen species (ROS), and hypoxia inducible factor-1 alpha (HIF-1α) signaling pathway in the protection against high glucose levels in brain endothelial and NT2 neuron-like cells. Rat brain endothelial cells (RBE4) treated with non-toxic concentrations of cyanide (≤1 μM; 1 h) exhibited an increase in ROS levels, particularly hydrogen peroxide (H_2O_2). Cyanide also induced a modest mitochondrial depolarization, an increase in oxygen consumption and a structural (smaller mitochondria) and spatial (perinuclear region) reorganization of mitochondrial network. The stabilization and nuclear activation of HIF-1α in the presence of cyanide were also observed, which resulted in an increase in vascular endothelial growth factor (VEGF), endothelial nitric oxide synthase (eNOS) and erythropoietin (EPO) protein levels reflecting an adaptive response. Importantly, preconditioning induced by cyanide protected brain endothelial cells against high glucose-mediated damage by the prevention of apoptotic cell death. In mitochondrial DNA-depleted NT2 (NT2 ρ0) cells, cyanide (0.1 μM) was unable to stimulate ROS production and, consequently, protect against glucotoxicity. Conversely, in NT2 cells, the parental cells with functional mitochondria, cyanide significantly increased ROS levels protecting against high glucose-induced neuronal cell loss and activation of caspase-3. The free radical scavenger N-acetyl-L-cysteine and the specific HIF-1α inhibitor 2-methoxyestradiol completely abolished the protective effects of cyanide preconditioning. Altogether our results demonstrate that mitochondrial preconditioning induced by cyanide triggers a protective response mediated by mitochondrial ROS and HIF-1α activation and signaling, which render brain endothelial and neuronal cells resistant against glucotoxicity.

Introduction

Diabetes mellitus is one of the most prevalent metabolic disorders that affects approximately 250 million people worldwide (Cole et al., 2007) and is associated with cognitive deterioration and changes in cerebral anatomy in humans (Biessels et al., 2006, 2008). Indeed, mild-to-moderate impairments of cognitive function have been reported in both type 1 and type 2 diabetic patients (Awad et al., 2004; Biessels et al., 2008). Type 1 diabetes, characterized by a deficit in the production of insulin by the pancreatic β cells, increases cognitive dysfunction and decreases the speed of mental processing (Brands et al., 2005). Type 2 diabetes, which presents as major pathological features peripheral insulin resistance and chronic hyperglycemia, is related with a faster rate in the decline of cognition in comparison with the general population (Allen et al., 2004). In addition, hemoglobin A1c level, a marker of the long-term hyperglycemia, was shown to correlate with cognitive decline in humans (MacLullich et al., 2004). In fact, chronic hyperglycemia has also been proposed to be one of the determinants of diabetes-related cognitive dysfunction by inducing structural and neurochemical abnormalities in the brain and thus, leading to the development of diabetic end-organ damage to the brain (Biessels et al., 2002; Gispen and Biessels, 2000; Kumagai, 1999; Mankovsky et al., 1996).

Preconditioning is a well accepted phenomenon, in which small doses of noxious stimulus are required to afford robust protective responses against future injury (Correia et al., 2010a). Although the molecular mechanisms underlying the induction and maintenance of preconditioning-induced brain tolerance are complex and remain largely unclear, mitochondrial-centered mechanisms have been proposed to be critical mediators of the preconditioning response

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(Busija et al., 2008). In fact, compelling evidence indicates that a slight rise of mitochondrial reactive oxygen species (ROS) triggers preconditioning-mediated brain tolerance, suggesting a role for mitochondria on endogenous neuroprotection (Correia et al., 2010a; Dirnagl et al., 2009; Jou, 2008). Conversely, the induction of the hypoxia signaling pathway with the concomitant stabilization and transcriptional activation of the transcription factor hypoxia-inducible factor-1α (HIF-1α) has emerged as one of the major cellular pathways responsible for preconditioning-induced neuroprotection (Sharp et al., 2004). HIF-1 is a heterodimeric protein composed of a constitutively expressed HIF-1β subunit and an inducible HIF-1α subunit. Under hypoxic conditions, HIF-1α translocates to the nucleus and recruits HIF-1β, modulates the expression of a wide range of protective genes involved in angiogenesis, metabolism, apoptosis and cell survival, including erythropoietin (EPO), vascular endothelial growth factor (VEGF) and endothelial nitric oxide synthase (eNOS) (Correia and Moreira, 2010). Additionally, HIF-1α activation seems to be strictly bound to mitochondrial function. Indeed, under hypoxic conditions, mitochondria act as oxygen sensors and convey signals to HIF-1, mitochondrial ROS being the putative signaling molecules between a cellular O_2-sensor and HIF-1 (Correia et al., 2010a, b).

Given the wide scientific evidence that highlights mitochondria as the key regulators of preconditioning (Correia et al., 2010a, b; Correia and Moreira, 2010), we hypothesized that mitochondrial preconditioning induced by cyanide (hereafter called cyanide preconditioning) may afford protection against glucotoxicity by modulating mitochondrial function and network organization and induction of HIF-1α signaling pathway. In the present study, we demonstrated that cyanide preconditioning is effective in protecting both brain endothelial and NT2 neuron-like cells against high glucose-induced damage. Additionally, the cytoprotective effects of cyanide preconditioning are reliant on functional mitochondria, mitochondrial ROS generation and induction of HIF-1α signaling pathway. Elucidation of the role of the mitochondrial ROS and HIF-1α in the protective mechanisms triggered by preconditioning may offer new avenues for the treatment of diabetes-associated neuronal and endothelial dysfunction.

Material and methods

Reagents

Fetal bovine serum (FBS), geneticin, HAM's F-10 and Mem-alpha medium with Glutamax-1 (α-MEM) were purchased from Gibco-Invitrogen (Grand Island, NY). Anti-β-actin antibody, basic Fibroblast Growth Factor (bFGF), D-glucose, N-acetylcysteine, potassium cyanide and anti-α-Tubulin antibody were obtained from Sigma (St. Louis, MO, USA). 2′-7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) and dihydrorhodamine 123 were obtained from Molecular Probes (Eugene, OR, USA). Rat tail collagen was purchased from Roche Diagnostics (Mannheim, Germany). 96-well Oxygen biosensor plates were purchased from BD Biosciences (San Jose, CA, USA). Anti-eNOS, anti-EPO and anti-TOM 20 antibodies were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Anti-HIF-1α antibody and anti-MTCO1 antibody were purchased from Abcam (Cambridge, UK). N-acetyl-Asp-Glu-val-Asp-P-milnoanilid (Ac-DEVD-*p*NA) and anti-VEGF antibody were obtained from Calbiochem, Merck KGaA (Darmstadt, Germany). Anti-Bax antibody was obtained from Cell Signaling (Danvers, MA, USA). All the other chemicals were of the highest grade of purity commercially available.

Cell culture

Rat brain endothelial cell line (RBE4) was kindly provided by Dr. Jon Holy (University of Minnesota, Duluth, USA). RBE4 cells were maintained as monolayer cultures in collagen-coated T-75 flasks in a humidified atmosphere of 5% CO_2–95% air at 37 °C. RBE4 cells were grown in α-MEM:Ham's F-10 nutrient mixture (1:1), supplemented with 10% FBS, 1 ng/ml bFGF and 300 μg/ml Geneticin (Roux et al., 1994). The medium was changed every 2–3 days until cells reached confluence. Human teratocarcinoma NT2 cells, a committed neuronal precursor cell line, containing mitochondrial DNA and NT2 cells depleted of mitochondrial DNA (NT2 ρ0) were grown in T-75 flasks in Optimen Medium containing 10% heat inactivated fetal calf serum, penicillin (50 U/ml), and streptomycin (50 μg/ml); the media for the NT2 ρ0 cells was further supplemented with uridine (50 μg/ml) and pyruvate (200 μg/ml) (Swerdlow et al., 1997). Both cell lines were maintained in a humidified atmosphere of 5% CO_2–95% air at 37 °C. The medium was changed every 2–3 days until cells reached confluence.

For Alamar blue, rhodamine 123 and ROS measurements, cells were plated in 48-well plates at a density of 0.5×10^5 cells/well. For LDH release and immunocytochemistry, cells were plated in 12-well plates at a density of 2×10^5 cells/well. For caspase enzyme activities, cells were plated in 6-well plates at a density of 5×10^5 cells/well. For nuclear and whole cell extracts, cells were plated in 10 cm Petri dishes at a density of 1×10^6 cells/dish.

Cell treatments

Cells were exposed to cyanide during 1 h at 37 °C. In some experiments (cell viability, ROS levels and caspase 3 activity) cells were pre-treated with 200 μM of N-acetylcysteine (NAC) or 5 μM of 2-methoxyestradiol (2-ME2) for 24 h and 30 min, respectively, before cyanide exposure. Concerning the experiments with high glucose levels, cell lines were pre-treated or not with cyanide and then washed with PBS and exposed to 30 mM of D-glucose for 12 h. Osmotic controls were done with 30 mM mannitol.

Assessment of cell viability

Cell viability was determined using the Alamar Blue assay. After the incubation period, the cells' medium was aspired and replaced by culture medium containing 10% (v/v) Alamar blue. After 1–2 h incubation at 37 °C, the supernatant was collected and the absorbance was measured at 570 nm and 600 nm using a microplate reader (SpectraMax Plus ^{384}, Molecular Devices) (Neves et al., 2006). Cell viability (% of control) was calculated according to the formula $(A_{57}-A_{600})$ of treated cells $\times 100/(A_{570}-A_{600})$ of control cells.

Measurement of lactate dehydrogenase (LDH) release

LDH activity was measured spectrophotometrically in the cell culture medium and cell lysates as described by Bergmeyer and Bernt (1974), which follows the conversion of NADH to NAD^+ at 340 nm. LDH release was expressed as the percentage of total LDH released into the medium.

Measurement of intracellular reactive oxygen species (ROS)

Intracellular ROS levels were quantified using the probe 2′-7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA). This probe is accumulated by cells and hydrolyzed by cytoplasmic esterases to become 2′-7′-dichlorodihydrofluorescein, which then reacts with ROS to generate the fluorescent product 2′-7′-dichlorofluorescein (DCF). Cell lines exposed or not to cyanide, in the presence or absence of NAC, were loaded with 20 μM $DCFH_2$-DA in sodium medium containing 132 mM NaCl, 4 mM KCl, 1.2 mM NaH_2PO_4, 1.4 mM $MgCl_2$, 6 mM glucose, 10 mM Hepes-Na, 1 mM $CaCl_2$, pH 7.4, for 30 min at 37 °C. After $DCFH_2$-DA incubation, cells were washed and sodium medium was added. Fluorescence was monitored for 1 h, at 37 °C, using a Spectramax GEMINI EM fluorocytometer (Molecular Devices), with excitation and emission wavelengths corresponding to 480 and