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Original Contribution

Insulin neuroprotection against oxidative stress in cortical neurons—Involvement of uric acid and glutathione antioxidant defenses

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Abstract

In this study we investigated the effect of insulin on neuronal viability and antioxidant defense mechanisms upon ascorbate/Fe^{2+}-induced oxidative stress, using cultured cortical neurons. Insulin (0.1 and 10 μM) prevented the decrease in neuronal viability mediated by oxidative stress, decreasing both necrotic and apoptotic cell death. Moreover, insulin inhibited ascorbate/Fe^{2+}-mediated lipid and protein oxidation, thus decreasing neuronal oxidative stress. Increased 4-hydroxynonenal (4-HNE) adducts on GLUT3 glucose transporters upon exposure to ascorbate/Fe^{2+} were also prevented by insulin, suggesting that this peptide can interfere with glucose metabolism. We further analyzed the influence of insulin on antioxidant defense mechanisms in the cortical neurons. Oxidative stress-induced decreases in intracellular uric acid and GSH/GSSG levels were largely prevented upon treatment with insulin. Inhibition of phosphatidylinositol-3-kinase (PI-3K) or mitogen-induced extracellular kinase (MEK) reversed the effect of insulin on uric acid and GSH/GSSG, suggesting the activation of insulin-mediated signaling pathways. Moreover, insulin stimulated glutathione reductase (GRed) and inhibited glutathione peroxidase (GPx) activities under oxidative stress conditions, further supporting that insulin neuroprotection was related to the modulation of the glutathione redox cycle. Thus, insulin may be useful in preventing oxidative stress-mediated injury that occurs in several neurodegenerative disorders.

Keywords: Cortical neurons; 4-HNE; Glutathione; Insulin; Oxidative stress; Uric acid

Introduction

In the central nervous system (CNS), insulin was reported to be involved in the regulation of brain metabolism [1,2], neuronal growth, and differentiation [3,4] or neuromodulation [5,6]. Insulin synthesized in pancreatic β-cells can be transported into the brain either through the blood–brain barrier via a receptor-mediated process or by direct entry at the area postrema, a circumventricular region that lacks the blood–brain barrier [7]. However, recent evidences suggest that insulin can be synthesized in pyramidal neurons, such as those from the hippocampus, prefrontal cortex, enthorhinal cortex, and olfactory bulb, but not in glial cells [8]. The observation of preproinsulin I and II mRNA within rat fetal brain and in cultured neurons, and also insulin immunoreactivity in the endoplasmic reticulum, Golgi apparatus, cytoplasm, axon,

Abbreviations: BSA, bovine serum albumin; *t*-BHP, *tert*-butylhydroperoxide; CNS, central nervous system; ERK, extracellular signal-related kinase; DNPH, 2,4-dinitrophenylhydrazine; DTT, 1,4-dithiotreitol; GLUT, glucose transporter; GPx, glutathione peroxidase; GRed, glutathione reductase; GSH, reduced glutathione; GSK-3β, glycogen synthase kinase-3β; GSSG, oxidized glutathione; GST, glutathione S-transferase; 4-HNE, 4-hydroxynonenal; IR, insulin receptor; LDH, lactate dehydrogenase; MEK, mitogen-induced extracellular kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NEM, *N*-ethylmaleimide; OPT, *ortho*-phthaldialdehyde; PI, propidium iodide; PI-3K, phosphatidylinositol-3-kinase; PBS, phosphate-buffered saline; PMSF, phenylmethanesulfonyl fluoride; PVDF, polyvinylidene difluoride; ROS, reactive oxygen species; SBTI, soybean trypsin inhibitor; TBARS, thiobarbituric acid-reactive substances; TBS, Tris-buffered saline; TCA, trichloroacetic acid.

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dendrites, and synapses of neuronal cells, further suggested that insulin can be synthesized in the brain [3,9,10]. Moreover, insulin receptor (IR) expression appears to be widely detected in brain, mainly in the olfactory bulb, hypothalamus, cerebral cortex, and hippocampus (for review, see [8,11]).

Insulin has been also suggested to have a neuroprotective role. Insulin was reported to protect against serum deprivation-induced neuronal death [12,13] and cerebral brain damage during ischemia [14]. Both conditions are related to neuronal death and oxidative stress, which results from the imbalance between production of reactive oxygen species (ROS) and antioxidant protection. The high content of easily oxidizable polyunsaturated fatty acids, transition metals and oxygen consumption and poor antioxidant protective defenses render neuronal cells more vulnerable to ROS, leading to the oxidation of lipids, proteins and DNA [15,16]. Thus, neuronal oxidative damage has been implicated in cell death by necrosis and apoptosis (for review, see [17]), occurring in several neurodegenerative disorders, namely Alzheimer's disease (AD) [18,19].

Previous studies performed in our laboratory showed that immunoreactive insulin is present in rat brain synaptosomes and that glucose metabolism may provide a signal, through glycolysis, for insulin release in the brain [2]. More recently, we found that insulin prevented ascorbate/Fe^{2+}-induced decrease in [^{3}H]glutamate and/or [^{3}H]GABA synaptosomal uptake under oxidative stress in normal and diabetic rats, suggesting a fundamental role for insulin in regulating neurotransmission under stress conditions, with a particular emphasis for GABA [20,21].

Although previous studies described the protection by insulin against serum-deprivation-induced neuronal apoptosis [12,13] and serum-free-induced necrosis [13], little is known about the role of insulin against oxidative stress-mediated neuronal injury. Thus, the aim of the present work was to investigate the neuroprotective effect of insulin in cortical neurons submitted to ascorbate/Fe^{2+}-induced oxidative stress. We found that insulin decreased lipid and protein oxidation, thus preventing neuronal death. Furthermore, insulin induced changes in uric acid and reduced glutathione, which involved the activation of phosphatidyl inositol-3-kinase (PI-3K)/Akt and extracellular signal-related kinase (ERK) signaling pathways.

Material and methods

Materials

Phenol red, penicillin/streptomycin, trypsin, DNase, soybean trypsin inhibitor (SBTI), insulin from porcine pancreas, ascorbic acid, ferrous sulfate ($FeSO_4$), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), propidium iodide (PI), thiobarbituric acid (TBA), 1,4-dithiotreitol (DTT), phenylmethanesulfonyl fluoride (PMSF), chymostatin, pepstatin A, leupeptin, antipain, Tween 20, reduced and oxidized glutathione (GSH and GSSG, respectively), *N*-ethylmaleimide (NEM), *ortho*-phthaldialdehyde (OPT), glutathione reductase (GRed), β-nicotinamide adenine dinucleotide reduced form (β-NADH), β-nicotinamide adenine dinucleotide phosphate reduced form (β-NADPH), and *tert*-butylhydroperoxide (*t*-BHP) were purchased from Sigma Chemical Co. (St. Louis, MO). Neurobasal medium and B-27 supplement were obtained from Gibco (Paisley, Scotland, UK). Hoechst 33342 was purchased from Molecular Probes (Leiden, The Netherlands). Polyvinylidene difluoride (PVDF) Hybond-P membranes, anti-mouse and anti-rabbit secondary antibodies, and protein A Sepharose CL-4B were purchased from Amersham Biosciences (Little Chalfont, UK). Mouse anti-α-tubulin monoclonal antibody was obtained from Zymed (San Francisco, CA). Rabbit anti-GLUT3 polyclonal antibody was purchased from Chemicon International (Temecula, CA). Rabbit polyclonal anti-HNE-Michael adducts (reduced), trichloroacetic acid (TCA), and PD98059 were from Calbiochem (Darmstadt, Germany). Polyacrylamide was from BioRad (Hercules, CA). Spin-X centrifuge tube filters used in immunoprecipitation were from Costar (New York). The enzymatic kit PAP 150 was purchased from BioMérieux (Lyon, France). Wortmannin and LY294002 were kindly given by Alomone Labs (Jerusalem, Israel).

All other reagents were of the highest grade of purity commercially available.

Primary culture of cortical neurons

Neuronal primary cultures were prepared from brains of 15- to 16-day-old Wistar rat embryos. Pregnant rat females obtained from our local colony (Animal Facilities at the Faculty of Medicine, University of Coimbra, Coimbra) were sacrificed by cervical delocalization. The brain cortices were removed from embryos to 20 ml of sterile isolation medium containing: 120.9 mM NaCl, 4.8 mM KCl, 1.22 mM KH_2PO_4, 25.5 mM $NaHCO_3$, 13.0 mM glucose, 10.0 mM Hepes, and 1 ml phenol red 0.5%, pH 7.4, supplemented with 0.3% bovine serum albumin (BSA). After a rapid centrifugation at 1000 rpm in a Sigma 3K10 centrifuge, the supernatant was removed and the pellet resuspended in 20 ml of BSA-supplemented isolation medium containing 0.02% trypsin and 40 μg/ml DNase, and trypsinized for 10 min at 37°C. Then, a solution containing 20 ml of BSA-containing isolation medium, supplemented with 0.052% SBTI and 40 μg/ml DNase, was added. The mixture was centrifuged for 5 min at 1000 rpm, and the resulting supernatant was discarded. The pellet was resuspended and mechanically dissociated in a solution containing 16.8 ml of the isolation medium supplemented with BSA and 3.2 ml of the solution used in the previous step, and then centrifuged for 5 min at 1000 rpm. After dissociation, the cells were resuspended in Neurobasal medium supplemented with 2% B-27, 0.2 mM glutamine, 100 units/ml penicillin, and 100 mg/ml streptomycin and cultured for 6 days in 95% air and 5% CO_2.