Chemico-Biological Interactions 242 (2015) 396–406

Contents lists available at ScienceDirect

Chemico-Biological Interactions

journal homepage: www.elsevier.com/locate/chembioint

Role for the PI3K/Akt/Nrf2 signaling pathway in the protective effects of carnosic acid against methylglyoxal-induced neurotoxicity in SH-SY5Y neuroblastoma cells

Marcos Roberto de Oliveira [a, *], Gustavo Costa Ferreira [b], Patrícia Fernanda Schuck [c], Simone Morelo Dal Bosco [d]

[a] Programa de Pós-Graduação em Química, Departamento de Química (DQ), Instituto de Ciências Exatas e da Terra (ICET), Universidade Federal de Mato Grosso (UFMT), Av. Fernando Corrêa da Costa, 2367, CEP 78060-900, Cuiabá, MT, Brazil
[b] Laboratório de Erros Inatos do Metabolismo, Unidade Acadêmica de Ciências da Saúde, Universidade do Extremo Sul Catarinense (UNESC), Programa de Pós-Graduação em Ciências da Saúde, Criciúma, SC, Brazil
[c] Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro (UFRJ), Rio de Janeiro, RJ, Brazil
[d] Programa de Pós-Graduação em Biotecnologia, Centro Universitário UNIVATES, Lajeado, RS, Brazil

ARTICLE INFO

Article history:
Received 30 June 2015
Received in revised form
7 August 2015
Accepted 4 November 2015
Available online 12 November 2015

Keywords:
Carnosic acid
Methylglyoxal
Oxidative stress
Akt
Nrf2

ABSTRACT

Glycation, a process that occurs endogenously and generates advanced glycation end products (AGEs), presents an important role in cases of neurodegeneration, as for instance Alzheimer's disease (AD). Methylglyoxal (MG), a dicarbonyl compound, is the most potent inducer of AGEs, whose levels have been found increased in samples obtained from subjects suffering from AD. Moreover, MG induces protein cross-linking and redox impairment *in vitro* and *in vivo*. Carnosic acid (CA), a phenolic diterpene isolated from *Rosmarinus officinalis*, exerts protective effects in neuronal cells by increasing antioxidant defenses and detoxification systems. In the present work, we aimed to investigate whether there is a role for CA against MG-induced neurotoxicity. Data obtained here clearly demonstrate that CA pretreatment (1 μM for 12 h) caused cytoprotective effects and counteracted the damage elicited by MG in SH-SY5Y cells. CA inhibited loss of mitochondrial membrane polarity (MMP) and cytochrome c release from mitochondria, consequently blocking activation of pro-apoptotic caspase enzymes. Furthermore, CA alleviated MG-induced oxidative and nitrosative damage. CA prevented MG-dependent neurotoxicity by activating the PI3K/Akt/Nrf2 signaling pathway and the antioxidant enzymes modulated by Nrf2 transcription factor. Overall, the data presented here show the protective role of CA by its ability to counteract MG negative effects.

1. Introduction

Methylglyoxal (MG), a α-ketoaldehyde, is a reactive dicarbonyl compound derived from the glycolytic pathway and is also originated nonenzymatically through reactions of sugar fragmentation [1,2]. The levels of MG rise during conditions of hyperglycemia and/or impaired glucose metabolism because triose phosphates (glyceraldehyde-3-phosphate and dihydroxyacetone phosphate), the major precursors of MG endogenously, accumulate inside cells [3]. Kalapos estimated that 0.1–0.4% of the glycolytic flux results in the generation of MG [4]. During fasting or events of insulin resistance, the overproduction of acetoacetate may lead to increased MG production through the conversion of acetone to MG [5]. Additionally, decreased levels of reduced glutathione (GSH) or increased nitric oxide ($NO^{\bullet}$) production impair glyoxalase I, resulting in augmented amounts of MG [3,6]. Catabolism of some amino acids, as for instance glycine and threonine, also gives rise to MG [7]. Alternatively, MG is a degradation product resulting from foodstuff autoxidation, photodegradation, and heating/cooking [2,8–10]. Finally, cigarette smoking [11], water purification procedures [12], and soil contamination through MG absorbed from polluted air by rainwater may increase human exposure to MG [13].

MG is characterized as the most potent inducer of advanced glycation end products (AGEs) production, as for instance argpyrimidine, N-carboxyethyl-lysine, and (hydro)imidazolone derivatives [14,15]. MG may induce glycation reactions 20,000 times

* Corresponding author.
E-mail addresses: mrobioq@gmail.com, mrobioq@yahoo.com.br (M.R. de Oliveira).

http://dx.doi.org/10.1016/j.cbi.2015.11.003
0009-2797/

more readily than glucose [15]. Furthermore, MG elicits cell damage by impairing redox parameters (reactive oxygen and nitrogen species – ROS and RNS, respectively), by inducing cross-linking of proteins, and glycation [2,16]. MG metabolism is an important source of reactive species and free radicals during its metabolism inside mammalian cells. MG autoxidation and photolysis generate hydrogen peroxide (H_2O_2), superoxide anion radical ($O_2^{-\bullet}$), and hydroxyl radical ($^{\bullet}OH$) [17,18]. The conversion of aminoacetone, pyruvate, or acetol to MG leads to the generation of hydrogen peroxide (H_2O_2) [19,20]. Moreover, aminoacetone autoxidation produces MG and carbon-centered radicals and $O_2^{-\bullet}$ [4,21]. In addition, MG interferes with mitochondrial function by disrupting electron flux between the mitochondrial electron transfer chain components and increasing $O_2^{-\bullet}$ production in different cell types, including neurons [22–26]. MG also induces cell death in several cell types [27]. Indeed, MG plays a role in pathologies in which redox impairment and increased rates of cell death have been reported, including diabetes mellitus (DM), cardiovascular diseases, and neurodegeneration, including Alzheimer's disease (AD) and Parkinson's disease (PD) [2,27]. Actually, MG-derived AGEs content is increased in the brain and cerebrospinal fluid of AD patients [28–30]. Furthermore, there are high levels of MG-derived AGEs in plaques and neurofibrillary tangles, the hallmarks of AD [31].

Nuclear factor erythroid-2 related factor (Nrf2), a master regulator in the cellular response to oxidative stress and xenobiotics, binds to the antioxidant responsive element (ARE) and induces the expression of several antioxidant and detoxifying enzymes, such as glutamylcysteine ligase (γ-GCL), glutathione reductase (GR), glutathione peroxidase (GPx), mitochondrial superoxide dismutase (Mn-SOD), and glutathione S-transferase (GST), among others [32,33]. In the absence of substantial inducers, Nrf2 is found mainly in the cytosol interacting with Kelch-like ECH-associated protein 1 (Keap1) [34]. However, when the concentrations of reactive species, electrophiles, and/or xenobiotics increase, Nrf2 is released from the protein complex with Keap1 and migrates to the cell nucleus, where it binds to ARE [35]. Among the inducers of Nrf2 activation, bioactive compounds are of particular interest. Such molecules may be utilized in a dietary strategy, for example, to induce the expression of antioxidant enzymes by interacting with Nrf2, as previously reported [36].

Carnosic acid (CA) is a diterpene isolated from *Rosmarinus officinalis* and presents antioxidant, anti-inflammatory, and anticarcinogenic effects in different biological systems [37–41]. CA modulates GSH synthesis in SH-SY5Y cells [42] and increases brain-derived neurotrophic factor (BDNF) in SN4741 cells [37]. Wu et al. demonstrated that CA is able to protect SH-SY5Y cells against 6-hydroxydopamine (6-OHDA) neurotoxicity by a mechanism that involves up-regulation of antioxidant enzymes [41]. Thus, CA may play a protective role in cases of neurodegeneration, as for instance AD. With this in mind, we investigated here whether a pretreatment with CA would exert beneficial effects on MG-induced SH-SY5Y toxicity. We have focused on redox parameters and apoptosis induced by MG.

2. Material and methods

2.1. Materials

Plastic material necessary for cell culture was purchased from Corning, Inc (NY, USA) and Beckton Dickson (NJ, USA). Culture analytical grade reagents were obtained from Sigma (MO, USA). All other chemicals and assay kits that were utilized in the present work were obtained as described below.

2.2. Cell culture and chemical treatment

Human dopaminergic neuroblastoma cell line (SH-SY5Y) was purchased from American Type Culture Collection (Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM)/F-12 HAM nutrient medium (1:1 mixture) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, penicillin (1000 units/mL), streptomycin (1000 μg/mL), and amphotericin B (2.5 μg/mL) in a 5% CO_2 humidified incubator at 37 °C. Cells were cultured until a confluence of 80–90% was achieved and then trypsinized. The cell culture medium was replaced every 2–3 days. All experiments were performed at cell confluence of 70–80% between passages 26 and 33. The cells were plated at an appropriate density according to the different experimental protocols utilized in this work.

In this experimental model, dopaminergic toxicity was induced by methylglyoxal (MG) at 500 μM as previously reported [43–45]. Depending on the objective, CA was utilized at concentrations ranging from 0.1 to 2 μM for different periods. To test the effects of a pretreatment with CA against the deleterious consequences triggered by MG, CA (dissolved in DMSO) was added to the incubation medium 12 h before MG. Specific concentrations and periods of treatment are indicated in the figure legends. The results are presented as the mean ± SEM of three or five independent experiments each done in triplicate.

2.3. Analyses of cellular viability, cytotoxicity, and apoptosis-related parameters

Cellular viability was assayed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay according to [46]. Briefly, the cells were plated onto 96-well plates and when the culture reached 70–80% confluence, the culture medium was removed and the treatments were applied. After the treatment of the cells with the desirable drug concentrations and incubation time, 20 μL of MTT solution (5 mg/mL sterile stock solution) was added to each well at a final concentration of 0.5 mg/mL. The cells were left for 4 h at 37 °C in a humidified atmosphere of 5% CO_2. Then, the medium was carefully removed and 100 μL DMSO for 30 min was utilized to dissolve the formazan crystals. The optical density of the wells was measured at 570 nm in a plate reader (Molecular Devices, CA, USA). Lactate dehydrogenase (LDH) leakage assay was performed in order to analyze membrane integrity according to the manufacturer instructions (CytoTox 96-NonRadioactive Cytotoxicity Assay, Promega).

Caspase-9 enzyme activity was quantified through the analyses of the cleavage of substrate LEHD-AFC (AFC: 7-amino-4-trifluoromethyl coumarin) by using a fluorimetric assay kit (excitation at 400 nm, emission at 505 nm; Abcam, MA, USA). Caspase-3 enzyme activity was measured by utilizing an assay kit (Sigma, MO, USA) in which the hydrolysis of acetyl-DEVD-AMC (AMC: 7-amino-methylcoumarin) results in free AMC, whose levels were read in a fluorescence plate reader (Molecular Devices, USA) as follows: excitation at 360 nm and emission at 460 nm.

To detect DNA fragmentation in cell lysates, the cells were incubated with 5′-bromo-2′-deoxy-uridine (BrdU) in order to label nuclear DNA. The BrdU-labeled DNA fragments are released from the cells to the cytoplasm during apoptosis and into cell culture during necrosis. We measured here cytoplasmic BrdU-labeled DNA fragments by an ELISA kit according to instructions of the manufacturer (Roche, Germany). After incubations with specific reagents, the samples were read at 450 nm (reference wavelength 690 nm) in a plate reader (Molecular Devices, CA, USA).