Biomedicine & Pharmacotherapy 108 (2018) 15–26

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

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

Original article

Amelioration of oxidative stress in differentiated neuronal cells by rutin regulated by a concentration switch

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ARTICLE INFO

Keywords:
Neurodegeneration
Oxidative stress
Phytochemicals
ROS
Rutin

ABSTRACT

Increasing studies have implicated superfluous production of reactive oxygen species (ROS) as a significant factor in the progress of neurodegenerative disorders ranging from ischemic stroke to amyotrophic lateral sclerosis. The possible mechanisms relating to oxidative stress and neurodegeneration are yet to be thoroughly understood. Rutin, a flavonoid, has been well documented for its beneficial and pharmacological activities against diverse targets. However, the mechanism involved in the beneficial effects of rutin against neurodegeneration still remains unclear. Our study investigates the concentration switch effects of rutin on differentiated human neuroblastoma cells (IMR32) *in vitro* to unveil the possible mechanism of its action. IMR32 cells were differentiated using retinoic acid and challenged with different doses of rutin for 24 h duration to study the influence of ROS on differentiated neuronal cells and ROS-mediated apoptosis. The study showed that the high (100 μM) and low (100 nM and 10μM) rutin concentrations significantly avert ROS generation by two different mechanisms, by enhancing apoptosis through the modulation of levels of Bcl2, Caspase-3, survivin and its antioxidant activity via stress-related proteins, JNK and p38 MAPK. Our study suggests that rutin is a multi-targeted therapeutic and preventive agent that may act as an adjuvant complementary therapeutic molecule to treat oxidative stress-mediated neurodegeneration.

1. Introduction

Neurodegenerative disorders are fast becoming the primary health burden among the aged population worldwide [1,2]. They are characterized by the gradual neuronal or myelin sheath destruction accompanied by the loss of motor or cognitive abilities. Globally, Parkinson's disease (PD) ranks as the second most incurable neurodegeneration condition [3,4] while Alzheimer's disease (AD) ranks as the sixth most common clinically recognized dementia that affects 1–2% of the aged population [5–7]. The molecular and cellular basis like aggregated protein deposition, impaired mitochondrial function, neuroinflammation, oxidative stress and activation of apoptotic factors along with aging, are the hallmarks of neurodegeneration [8]. Despite the greater understanding of the pathogenesis leading to neurodegeneration, the treatment for most of these conditions is still lacking.

Reactive oxygen species (ROS)-mediated oxidative stress and chronic inflammation accompanied by cell apoptosis are the major players in the genesis and progression of neurodegeneration [9–12]. ROS may not be the triggering factor for neurodegenerative diseases but can exacerbate disease progression through oxidative damage and interaction with mitochondria causing cellular dysfunctions [13]. In addition, the disproportion between the fragile oxidant and antioxidant ratio in the cells coupled with the high metabolic activity makes the neuronal cells more vulnerable to oxidative stress which in turn triggers neuronal cell damage and cell death [14,15]. Currently, pharmacotherapy using cholinesterase inhibitors, memantine, cognitive therapy, immunotherapy, disease-modifying therapies and combination therapy are employed to manage neurodegenerative disorders [16]. However, these strategies are all symptom-oriented and are also associated with severe side effects, limited efficacy and only partially suppress the disease progression [17]. There is no effective curative measure for regeneration of neuronal cells and gain of cognitive function in neurodegenerative disease patients. In this context, alternative and

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https://doi.org/10.1016/j.biopha.2018.09.021
Received 15 June 2018; Received in revised form 18 August 2018; Accepted 4 September 2018

complementary medicine have garnered attention as they show promise as sources of new pharmacologically active molecules with superior therapeutic efficacy and a history of prolonged traditional use [18].

Dietary intake of phytochemicals and their effects on health, in specific to attenuation of generation of ROS by native antioxidants from various herbs, green teas, and spices have been well documented [19]. Earlier studies have shown that flavonoids were found to possess remarkable biochemical and pharmacological activities including neuroprotective effects. It has been reported that flavonoids like quercetin, myricetin, fisetin, morin, kaempferol, genistein, etc., can improve cognitive processing through enhancement of synaptic plasticity, neuroprotection, and aiding neuronal differentiation as well as promoting long-term potentiation [20,21]. Rutin (RUT) alternatively referred as Vitamin P or Rutoside, is one such polyphenolic flavonoid found abundantly in citrus fruits. Chemically, it is a glycoside combination of quercetin and rutinose and manifests numerous pharmacological properties like the antidepressant, neuroinflammatory, anti-allergic, anti-arthritic, immunomodulatory, antiproliferative and anti-carcinogenic properties [22]. It also exhibits cardioprotective, nephroprotective and neuroprotective functions [23,24]. It is an efficient free radical scavenger and has been demonstrated to act as a neuroprotectant in reperfusion-induced cerebral injury and ischemic animal models [25–27].

RUT effectively inhibits the activation of proinflammatory cytokines and microglia, which are the major players involved in neuroinflammation [28]. Koda et al. have reported that RUT acts as a neuroprotectant in rodent models treated with trimethyltin to induce spatial memory defects by damaging hippocampal neurons in the CA3 subregion implicated in learning process [29]. *in vitro* studies employing Caco-2 cells have shown that RUT was able to reverse mitochondrial damage, oxidative stress and apoptosis induced by indomethacin [30]. It also was shown to overcome oxidative stress-induced cellular damage in neurodegenerative disorders such as PD and AD [30,31]. Wang et al. reported that RUT reduced the β-amyloid-induced oxidative stress and decreased nitric oxide and inflammation-associated cytokine production [32]. Several reports have indicated that RUT may possess prooxidant properties at particular concentrations that may transform it into a cytotoxic agent [33]. Therefore, the influence of RUT on neuronal toxicity and oxidative stress remains inconclusive. Greater comprehension of the mechanisms underlying the dose-dependent effects of RUT in differentiated neuronal cells will aid to design effective treatment regimens to mitigate neurodegeneration arising due to excessive ROS. In this study, we proposed to assess the diverse function of RUT on neurotoxicity and oxidative stress in mature neuronal cells.

2. Materials and methods

2.1. Materials

Rutin, All-trans retinoic acid (ATRA), 2′,7′-Dichlorofluorescin diacetate (DCFA-DA), and JC-1 dye (5,5′,6,6′-tetraethyl benzidazolyl carbocyanine iodide) were procured from Sigma Chemicals Ltd. (USA). Dulbecco's modified Eagle medium (DMEM), and fetal bovine serum (FBS) were purchased from Gibco (MD, USA). Antibodies against microtubule-associated protein 2 (MAP2), B-cell lymphoma-2 (Bcl-2), caspase-3, survivin, c-jun N-terminal kinase (JNK), p38 mitogen activated protein kinase (p38MAPK) and β-actin and secondary antibody, Horse radish peroxidase (HRP)-conjugated goat anti-rabbit IgG (immunoglobulin G) were purchased from Cell Signaling Technology (MA, USA). Streptomycin and penicillin, calcein and ethidium bromide were obtained from Invitrogen (USA). Nitrocellulose membrane was purchased from Bio-Rad (USA). Molecular and cell culture grade chemicals used in the study were procured from Merck, SRL and Himedia Pvt. Ltd., India.

2.2. Methods

2.2.1. Cell culture

Human neuroblastoma cell line, IMR32 was obtained from National Centre for Cell Sciences, Pune, India. IMR32 cells were cultured to attain confluence in T-75 flasks using DMEM supplemented with L-glutamine and sodium pyruvate, which were added along with 10% FBS, 1% streptomycin (100 mg/mL) and penicillin (100U/mL) in carbon dioxide (CO_2) incubator. 2.5×10^4 cells/cm^2 were seeded and cultured using differentiation medium (DMEM, ATRA and 1% FBS) for 10 days. RUT of 5 mM concentration dissolved in dimethyl sulfoxide was employed as a stock and used for further experiments with appropriate dilutions. Control cells received the vehicle alone (0.01% DMSO).

2.2.1.1. IMR32 cell differentiation. Cells were cultured in a six-well plate and undifferentiated IMR32cells were maintained simultaneously as control. ATRA was added to the other groups of cells after the second day when the cells attained confluent. Varying concentrations of ATRA ranging from 2 to 10 μM was given to the cells and the duration for the differentiation was optimized by regularly monitoring the cell morphology. Cells were imaged every two days using phase contrast microscope (Zeiss microscope, Germany) to optimize the time and concentration-dependent neuronal differentiation.

2.2.2. Dose-response effects of RUT on cell viability

Cytotoxicity was measured by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay which is based on the metabolic activity of the cell. Cell viability was assessed by MTS conversion to purple formazan by cellular dehydrogenase enzymes. In a 96 well plate, 1×10^4 cells per well were seeded, cultured for 24 h and then, the cells were challenged with RUT in a dose (1 nM to 1 mM)- and time (24 and 48 h)-dependent manner. After the specified time points, the MTS dye was added and incubated for 2–4 h in a CO_2 incubator. The cells were then lysed using 10% sodium dodecyl sulfate (SDS) and the optical density was measured at 564 nm using Tecan multimode reader (Infinite M200, Tecan, Austria). A similar study was performed in differentiated cells with the same concentration range after 6 days of ATRA exposure. Six independent experiments were performed similarly with four replicate wells for each analysis. Results are expressed as percentage cell viability relative to the control (undifferentiated IMR32 cells without RUT exposure).

2.2.3. Assessment of morphology, neurite length and analysis of differentiation marker

5×10^4 cells /well were cultured in a six-well cell culture plate and differentiated for 6 days using differentiation medium and then treated with 100 nM, 10 μM, and 100 μM RUT concentrations. Undifferentiated cells were also seeded at the same density and treated with 100 μM RUT and respective controls were maintained as required. The morphological changes have been imaged and represented after 24 h of RUT exposure. The dendrite length was quantitatively analyzed using Neuron Growth® software in ImageJ (v1.50i). Differentiation marker, MAP2 has been analyzed using Western blot as described in Section 2.2.7

2.2.4. Analysis of Oxidative stress through intracellular ROS levels

Intracellular ROS was assessed using DCFA-DA method by fluorescence microscopy. 5×10^4 cells per well were seeded and cultured onto the coverslips separately for differentiation and undifferentiation conditions and challenged with different doses of RUT for 24 h. After 24 h of RUT exposure, the cells were rinsed with 1X phosphate buffered saline (PBS), 20 μM DCFA-DA was added and kept in dark for 20 min. Finally, the cells were rinsed with 1X PBS and the fluorescence images were captured using Laser Scanning Confocal Fluorescence Microscopy (excitation, 485 nm; emission, 530 nm) (FV1000, Olympus, USA). Fluorescence intensity of ROS levels was quantified using ImageJ