Food and Chemical Toxicology 90 (2016) 160–170

Contents lists available at ScienceDirect

Food and Chemical Toxicology

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

Potential role of autophagy in smokeless tobacco extract-induced cytotoxicity and in morin-induced protection in oral epithelial cells

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ARTICLE INFO

Article history:
Received 28 October 2015
Received in revised form
9 February 2016
Accepted 11 February 2016
Available online 15 February 2016

Keywords:
Smokeless tobacco
Reactive oxygen species
Autophagy
Morin

ABSTRACT

Toxic components of STE induced serious, adverse human oral health outcomes. In the present study, we observed that STE was involved in oral toxicity by reducing the viability of human squamous epithelial cells, SCC-25, along with the simultaneous induction of both apoptosis and autophagic signaling. STE was also found to induce significant amount ROS generation in SCC-25 cells. The dietary flavonoid morin, found abundantly in a variety of herbs, fruits and wine, has been reported to attenuate ROS-induced pathogenesis including autophagy. In this study we designed three different treatment regimes of morin treatment, such as pre, co, and post – treatment of STE challenged SCC-25 cells. In all cases morin provided cytoprotection to STE challenged SCC-25 cells by augmenting STE induced ROS-dependent cytotoxic autophagy. Hence, morin is a potential option for antioxidant therapy in treatment of STE induced toxicity.

1. Introduction

Habit of using smokeless tobacco by people of different age groups has become a world wide concern for human health due to its increasing adverse effects. Use of smokeless tobacco (ST) in form of “Spit tobacco” and “chewing tobacco” causes severe toxicity in oral tissue of the tobacco users (Bates et al., 2003a). It has been reported that when the oral tissues are exposed to smokeless tobacco, either by snuff or chewing, it causes buccal mucosa injury and inflammation, which leads to snuff dippers lesion, also known as leukoplakia (Robertson et al., 1997). Several reports demonstrate that exposure to tobacco specific nitrosamine (TSNA) lead to oral epithelial cell dysplasia and cancer (Hoffmann et al., 1991). Researchers have shown that oral exposure to moist smokeless tobacco is associated with cancer of mouth, lip, nasal cavities, oesophagus and gut (Brinton et al., 1984; Johansson et al., 1989).

Apoptosis has been commonly reported as a potent mechanism of ST-induced cytotoxicity in a variety of cell lines such as, oral keratinocytes (Bagchi et al., 1999), macrophages (Lombard et al., 2010), lung and hepatic epithelial cells (Das et al., 2013), to mention a few. Thus apart from causing cancer, ST also results in significant cellular injury, which adversely affect the overall human health. In the previous report, we have demonstrated that tubulin-microtubule system, which constitute the cellular cytoskeleton and participate in variety of cellular processes (Das et al., 2013), act as a potent target for smokeless tobacco extract (STE) in epithelial cells. STE-treatment of lung and hepatic epithelial cells resulted in the disruption and simultaneous degradation of cellular microtubule network, whereas the other cytoskeleton protein actin remains unaffected (Das et al., 2013) Beside this, STE is reported to induce ROS/RNS in macrophages (Bagchi et al., 1995), and oral keratinocytes (Bagchi et al., 1999). All these factors might contribute to STE-induced apoptosis in mammalian cells.

Autophagy is an evolutionarily conserved cellular process, which leads to the degradation of cytosolic components and organelles, and has a significant implication in variety of physiological and pathophysiological events, such as starvation adaptation, intracellular protein and organelle clearance, development, anti-aging, elimination of microorganisms, cell death, tumor suppression, and antigen presentation to name a few (Mizushima, 2007). Autophagy plays a dual role in context to the cell survival as well as cell death. In the absence of stress, autophagy occurs at low basal level to maintain cellular homeostasis by degrading intracellular damaged proteins and organelles (He and Klionsky, 2009; Levine and Klionsky, 2004). But accumulating evidences suggest that, in the presence of ROS, autophagy acts as a cell death machinery

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http://dx.doi.org/10.1016/j.fct.2016.02.011
0278-6915/

(Filomeni et al., 2015; Lang et al., 2015; Tsujimoto and Shimizu, 2005; Wang et al., 2010). Although there are previous reports of STE-induced ROS generation in mammalian cells, STE-induced autophagy has not been reported. In the present study, we demonstrated for the first time that autophagy occurred in the very early stage of STE-treatment in human squamous epithelial cells, SCC-25. We investigated how STE modulated autophagy in SCC-25 cells in detail and observed that autophagy also contributes to STE-mediated cytotoxicity.

Morin (3,5,7,2′,4′-pentahydroxyflavone) is a natural yellowish bioflavonoid (Fig. 1), which is the constituent of many herbs, fruits and wine (Sreedharan et al., 2009). Morin was originally isolated from members of the Moraceae family of plants, which are extensively used as food and traditional herbal medicine (Zhang et al., 2011), exhibits different pharmacological properties, including antioxidant, xanthine oxidase inhibitor (Yu et al., 2006), anti-inflammatory (Fang et al., 2003), chemo-preventive (Kawabata et al., 1999), and anticancer properties (Kuo et al., 2007). Furthermore, it gives protection to cardiovascular cells (Kok et al., 2000), glomerular mesangial cells (Zeng et al., 1994), hepatocytes (Sivaramakrishnan et al., 2008), and neurons (Gottlieb et al., 2006) against ROS-induced oxidative damage. Morin is also reported to confer protection to primary hepatocytes against hyperglycemia-induced oxidative damage (Kapoor and Kakkar, 2012). Hence morin may have potential as an antioxidant therapy against ROS-induced disorders.

Since it is well established that ROS is the main etiological factor behind STE-mediated cellular/tissue damage, in the present study we aim to investigate the effectiveness of morin against STE-mediated cytotoxicity. We set up three different treatment conditions, such as pre-, co- and post-treatment with morin in STE-treated SCC-25 cells. Effect of morin on STE-induced apoptosis, autophagy and microtubule disruption in SCC-25 cells, were investigated in detail.

2. Materials and methods

2.1. Materials

Ham's F12 nutrient media and DMEM nutrient media (1:1 ratio) (supplemented with 1 mM L-glutamine), Penicillin-streptomycin, Amphotericin B, Trypsin-Versene (1×) and FBS were purchased from Hi Media. 5, 5'-dithiobis (2-nitrobenzoic acid) (DTNB), were purchased from SIGMA, USA. Mouse monoclonal anti-α-tubulin, anti-bax, anti-Nrf-2, anti- Beclin I, anti LC-III antibody, rabbit polyclonal anti-Bcl-2 antibody, goat polyclonal caspase-3 antibody, and annexin V-FITC apoptosis kit were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Human oral squamous epithelium carcinoma cell line (SCC-25) was generous gift from Dr Sushanta Roy Choudhury, IICB (Indian Institute of Chemical Biology), Kolkata, India. Bradford protein estimation kit was purchased from GeNei, India. All other chemicals and reagents were purchased from Sisco Research Laboratories, India.

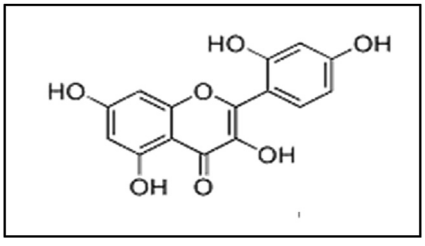

Fig. 1. Chemical structure of morin.

2.2. Preparation of aqueous extract of smokeless tobacco (STE)

According to the standard procedure of STE preparation published by (Das et al., 2013; Mitchell et al., 2010), we first added 50 ml PBS to 10 gm of commercially available smokeless tobacco and after that we incubated at 37 °C for 24 h under rotating condition. Consequently the water soluble components of smokeless tobacco were dissolved in PBS. Then we separated out undissolved materials of smokeless tobacco by filtration through whatman filter paper and subsequently 0.22 μ membrane filter paper, under sterile condition. The filtrate was then adjusted to pH 7 by using 1 M NaOH and subsequently lyophilized, we obtained 2.14 gm STE in powder form. Now we prepare stock concentration of STE by measuring 10 mg STE lyophilized powder and dissolved it in sterile 1 ml PBS and adder to the cells according to the experimental requirement. During the lypholization salt might precipitated down hence the doses of STE mentioned in the manuscript represent the relative concentration, where amount of salts are excluded from the total amount of STE preaparation and this expressed in μg/ml. During the course of treatment we checked the pH of the cell culture media and it was found to be neutral (around pH-7.2).

2.3. Cell culture and treatment

Human oral carcinoma cell line (SCC-25) were maintained in Ham's F12 nutrient media and DMEM nutrient media (1:1 ratio) also supplemented with 400 ng/ml hydrocortisone, 2.5 mM L-glutamine, all these media were additionally supplemented with 1 mM L-glutamine, 10% fetal bovine serum, 0.2% $NaHCO_3$, 1 mM penicillin, 1 mM streptomycin and 1 mM fungizone (pH 7.4). The cells were cultured at 37 °C maintain humidified atmosphere which containing 5% CO2. Cells were grown in tissue culture flasks until they were become 80% confluent before trypsinization with 1% Trypsin-EDTA and splitting.

2.4. Cytotoxicity assay

By using MTT (3-(4,5-dimethyl thiazolyl-2)-2,5-diphenyltetrazolium bromide) assay we evaluated STE-induced cytotoxicity in SCC-25 cells. At first cells were plated in 96-well culture plates (1×10^4 cells per well). After 36 h incubation, the cells were treated with STE for required time. After MTT (5 mg/ml) was dissolved in PBS and filter sterilized, then 20 μl of the prepared solution was added to each well and cells were incubated until a purple precipitate was visible. Then 100 μl of Triton-X was added and left the well in the dark for 2 h at room temperature. The absorbance was measured on an ELISA reader at a test wavelength of 570 nm and a reference wavelength of 650 nm. Percentage of cell viability was calculated by the following formula (Das et al., 2014):

$$\% \text{ inhibition} = (100 - (A_t/A_s) \times 100) \times \% \quad (1)$$

A_t and A_s indicated the absorbance of the test substances and solvent control, respectively.

2.5. Estimation of apoptotic cells

Apoptosis was measured with an annexin V-FITC apoptosis detection kit, following the published protocol (Das et al., 2012b). Cultured SCC-25 cells (1×10^5) were subjected to STE-treatment and then stained for 15 min at room temperature in the dark with fluorescein isothiocyanate (FITC)-conjugated annexinV (1 μg/ml) and propidium iodide (PI) (0.5 μg/ml) in a Ca^{2+}-enriched