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Original Contribution

Redox-dependent induction of antioxidant defenses by phenolic diterpenes confers stress tolerance in normal human skin fibroblasts: Insights on replicative senescence

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ABSTRACT

Mild stress-induced hormesis represents a promising strategy for targeting the age-related accumulation of molecular damage and, therefore, for preventing diseases and achieving healthy aging. Fruits, vegetables, and spices contain a wide variety of hormetic phytochemicals, which may explain the beneficial health effects associated with the consumption of these dietary components. In the present study, the induction of cellular antioxidant defenses by the phenolic diterpenes carnosic acid (CA) and carnosol (CS) were studied in normal human skin fibroblasts, and insights into the aging process at the cellular level investigated. We observed that CA and CS induced several cytoprotective enzymes and antioxidant defenses in human fibroblasts, whose induction was dependent on the cellular redox state for CS and associated with Nrf2 signaling for both compounds. The stress response elicited by preincubation with CS conferred a cytoprotective action against a following oxidant challenge with *tert*-butyl hydroperoxide, confirming its hormetic effect. Preincubation of normal fibroblasts with CS also protected against hydrogen peroxide-induced premature senescence. Furthermore, cultivation of middle passage normal human skin fibroblasts in the presence of CS ameliorated the physiological state of cells during replicative senescence. Our results support the view that mild stress-induced antioxidant defenses by CS can confer stress tolerance in normal cells and may have important implications in the promotion of healthy aging.

Introduction

Population aging was one of the most distinctive demographic events marking the twentieth century and will certainly remain an important tendency throughout this century [1,2]. The worldwide prolongation of the mean life expectancy has resulted in a rapid increase in the size of the elderly population, both in absolute numbers and as a proportion and, consequently, increased the incidence of age-related diseases [1]. As such, population aging presents new and serious medical, social, and financial challenges for modern societies [1,3].

Aging is characterized by the progressive accumulation of molecular damage, whose main cause is the inefficiency and failure of cellular maintenance and repair mechanisms. Therefore, mild stress-induced stimulation of these mechanisms has been recognized as a promising strategy to prevent age-related diseases and achieve healthy aging [4–6]. The process in which exposure to a low level of stress elicits adaptive beneficial responses that protect against subsequent exposure to severe stress is a phenomenon known as hormesis [7,8]. The paradigm for hormesis is physical exercise, which stresses the muscle cells but induces adaptation when practiced moderately. Indeed, moderate exercise is beneficial to health by increasing the physiological function of different organs. On the other hand, physical inactivity and arduous exercise decrease physiological function and increase the risk of diseases [9].

Fruits, vegetables, and spices contain a wide variety of hormetic phytochemicals, such as resveratrol, sulforaphane, and curcumin,

Abbreviations: ARE, antioxidant response element; CA, carnosic acid; CPD, cumulative population doubling; CS, carnosol; FTH1, ferritin heavy chain; GCL, glutamate cysteine ligase; GSH, glutathione; GST-P1, glutathione *S*-transferase P1; H_2O_2, hydrogen peroxide; HO-1, heme oxygenase-1; Keap1, Kelch-like ECH-associated protein; NAC, *N*-acetylcysteine; NQO1, NADP(H):quinone oxidoreductase-1; Nrf2, nuclear factor erythroid 2-related factor; ROS, reactive oxygen species; SA-β-gal, senescence-associated β-galactosidase; *tert*-BOOH, *tert*-butyl hydroperoxide; TXNRD1, thioredoxin reductase 1.

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that stimulate cell stress-induced maintenance and repair mechanisms and which may explain the health benefits associated with the consumption of these dietary components [10–16]. Many of these hormetic phytochemicals are inducers of the nuclear factor erythroid 2-related factor (Nrf2), a basic leucine zipper transcription factor that plays a key role in orchestrating the induction of several cytoprotective genes containing at least one antioxidant response element (ARE) within their promoters [17,18]. Under normal conditions, the Kelch-like ECH-associated protein (Keap1) forms a complex with cullin 3 (Cul3) and represses Nrf2 by presenting it for ubiquitination and proteasomal degradation. On stimulation, the highly reactive cysteine residues of Keap1 are modified, resulting in conformational changes that abrogate the capacity of Keap1 to repress Nrf2. Under these conditions, the transcription factor accumulates in the nucleus and, in heterodimeric combination with small Maf transcription factors, binds to ARE and recruits the basal transcriptional machinery to activate transcription of genes encoding stress-responsive and cytoprotective enzymes and related proteins [18–20].

Several studies on different species demonstrated that expression of Nrf2 and its target genes declines during aging and disease [21–24]. Indeed, the decline of Nrf2 transcriptional activity causes the age-related loss of glutathione synthesis, which adversely affects cellular thiol redox balance, leaving cells highly susceptible to different stresses [21]. In some animal models, such as *Drosophila* and *Caenorhabditis elegans*, Nrf2 orthologues have been associated with oxidative stress tolerance and aging modulation [24–27]. Therefore, the Nrf2 signaling pathway has been pointed out as a regulator of health span and its induction by hormetic phytochemicals may explain their health-promoting effects [14,26].

In the present study, carnosic acid (CA) and carnosol (CS), two phenolic diterpenes found in *Rosmarinus officinalis* (rosemary) that have been reported to activate the Nrf2/ARE signaling pathway [28–30], were used. Their ability to induce antioxidant defenses and cytoprotective enzymes, as well as to confer stress tolerance, was investigated in normal diploid human skin fibroblasts to better link the effects to the aging process at the cellular level. For that, both compounds were also studied using a model of stress-induced premature senescence and during replicative senescence.

Materials and methods

Chemicals and antibodies

Carnosic acid and carnosol were purchased from LGC standards (Teddington, UK). Dulbecco's modified Eagle's medium (DMEM), antibiotic-antimycotic solution, kinase inhibitors LY294002, PD98059, and SP600125, *tert*-butyl hydroperoxide (*tert*-BOOH), hydrogen peroxide (H_2O_2), anti-β-actin antibody (dilution 1:2000), and all other not specified reagents were from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) was obtained from Biochrom AG (Berlin, Germany), complete protease inhibitor cocktail from Roche (Penzberg, Germany), 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) from Apollo Scientific Ltd (Stockport, UK), 2′,7′-dichlorodihydrofluorescein diacetate from Molecular Probes (Eugene, OR, USA), and SYBR Gold nucleic acid gel stain was purchased from Invitrogen (Paisley, UK).

An SV Total RNA isolation system was purchased from Promega (Madison, WI, USA), iScript cDNA synthesis kit and SsoFast EvaGreen Supermix from Bio-Rad Laboratories (Hercules, CA, USA), primers from STAB VIDA (Caparica, Portugal), and TransAM Nrf2 kit from Active Motif (Carlsbad, CA, USA).

Antibodies against NQO1 (dilution 1:1000), ferritin (dilution 1:250), and histone H1 (dilution 1:250) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-GCLM antibody (dilution 1:500) developed by the Clinical Proteomics Technologies for Cancer was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biology (Iowa City, IA, USA). Anti-HO-1 (dilution 1:500) and anti-GST-Pi (dilution 1:1000) antibodies were from Enzo Life Sciences (Farmingdale, NY, USA), anti-caspase-3 (dilution 1:5000) from EMD Millipore Corporation (Billerica, MA, USA), and anti-Nrf2 (dilution 1:5000) from Novus Biologicals (Littleton, CO, USA). Finally, horse anti-mouse IgG-HRP and goat anti-rabbit IgG-HRP secondary antibodies were from Cell Signaling Technology (Beverly, MA, USA).

Cell culture and experimental conditions

Normal diploid adult human skin fibroblasts (ASF-2 cells) isolated from a breast biopsy specimen of a consenting young healthy Danish woman (aged 28 years) were used [31]. Cells were maintained in DMEM supplemented with 10% FBS, 1% antibiotic antimycotic solution, and 10 mM Hepes, at 37 °C in a humidified incubator containing 5% CO_2. Culture medium was renewed every 2 days until cells reached 90–95% confluence and then they were subcultivated. At each subcultivation, harvested cells were counted in a hemocytometer and the number of population doublings was calculated using the following equation: log10 [(number of cells harvested) / (number of cells seeded)] / log10 (2). The calculated population doublings were then added to the previous population doublings, to yield the cumulative population doublings (CPDs) [32]. All experiments were performed in low passage fibroblasts (cumulative population doublings between 15 and 25), except the replicative senescence experiments that were performed in middle passage fibroblasts (cumulative population doublings between 30 and 35), at a density of 50,000 cells/ml. CA and CS were dissolved in dimethyl sulfoxide (DMSO) at such concentrations that the final solvent concentration did not exceed 0.5% (v/v) when added to the cell culture medium. A similar concentration of DMSO (up to 0.5% v/v) was added to controls.

Glutathione content

Glutathione (GSH) content was determined by the DTNB-GSSG reductase recycling assay as previously described [33], with some modifications [34]. Briefly, after protein precipitation with 5-sulfosalicylic acid, samples were centrifuged and the resultant supernatants used for measurement of glutathione following the TNB formation at 415 nm and compared with a standard curve. The results were expressed as nanomole GSH per milligram of protein.

Total protein extraction

Total protein extracts were performed as previously described [12]. Briefly, cells were rinsed with PBS and then lysed in ice-cold lysis buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 2 mM EDTA, 1% v/v NP-40) containing 20 mM NaF, 20 mM Na_3VO_4, 1 mM PMSF, and 1 × complete protease inhibitor cocktail. Protein concentration was quantified using the DC protein assay (Bio-Rad Laboratories) and BSA used as protein standard.

Cytosolic and nuclear protein extraction

Cytosolic and nuclear protein extracts were prepared as previously described [12]. Briefly, cells were incubated with ice-cold hypotonic buffer (10 mM Hepes, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$) containing 0.5 mM DTT, 1 mM PMSF, and 1X complete protease inhibitor cocktail and then NP-40 was added to a final concentration 0.7% (v/v). The homogenate was centrifuged and the cytosolic supernatant harvested. The nuclear pellet was resuspended in ice-cold nuclear buffer (20 mM Hepes, pH 7.9, 0.4 M NaCl, 1 mM EDTA,