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Gamma-irradiation produces active chlorine species (ACS) in physiological solutions: Secoisolariciresinol diglucoside (SDG) scavenges ACS - A novel mechanism of DNA radioprotection

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ABSTRACT

Background: Secoisolariciresinol diglucoside (SDG), the main lignan in whole grain flaxseed, is a potent antioxidant and free radical scavenger with known radioprotective properties. However, the exact mechanism of SDG radioprotection is not well understood. The current study identified a novel mechanism of DNA radioprotection by SDG in physiological solutions by scavenging active chlorine species (ACS) and reducing chlorinated nucleobases.

Methods: The ACS scavenging activity of SDG was determined using two highly specific fluoroprobes: hypochlorite-specific 3′-(*p*-aminophenyl) fluorescein (APF) and hydroxyl radical-sensitive 3′-(*p*-hydroxyphenyl) fluorescein (HPF). Dopamine, an SDG structural analog, was used for proton ^{1}H NMR studies to trap primary ACS radicals. Taurine *N*-chlorination was determined to demonstrate radiation-induced generation of hypochlorite, a secondary ACS. DNA protection was assessed by determining the extent of DNA fragmentation and plasmid DNA relaxation following exposure to ClO^- and radiation. Purine base chlorination by ClO^- and γ-radiation was determined by using 2-aminopurine (2-AP), a fluorescent analog of 6-aminopurine. Results: Chloride anions (Cl^-) consumed >90% of hydroxyl radicals in physiological solutions produced by γ-radiation resulting in ACS formation, which was detected by ^{1}H NMR. Importantly, SDG scavenged hypochlorite- and γ–radiation-induced ACS. In addition, SDG blunted ACS-induced fragmentation of calf thymus DNA and plasmid DNA relaxation. SDG treatment before or after ACS exposure decreased the ClO^- or γ-radiation-induced chlorination of 2-AP. Exposure to γ-radiation resulted in increased taurine chlorination, indicative of ClO^- generation. NMR studies revealed formation of primary ACS radicals (chlorine atoms ($Cl^•$) and dichloro radical anions ($Cl_2^{-•}$)), which were trapped by SDG and its structural analog dopamine.

Conclusion: We demonstrate that γ-radiation induces the generation of ACS in physiological solutions. SDG treatment scavenged ACS and prevented ACS-induced DNA damage and chlorination of 2-aminopurine. This study identified a novel and unique mechanism of SDG radioprotection, through ACS scavenging, and supports the potential usefulness of SDG as a radioprotector and mitigator for radiation exposure as part of cancer therapy or accidental exposure.

1. Introduction

Active chlorine species (ACS) include chlorine-containing molecules in oxidation states other than −1. In physiological solutions, ACS are represented by molecules in oxidation states 0 and +1, namely, chlorine atoms ($Cl^•$), chlorine molecules (Cl_2), dichloro radical anions ($Cl_2^{-•}$), hypochlorous acid (HOCl) and hypochlorite anions (ClO^-), which are formed by the oxidation of chloride anion Cl^- [1–7]. Among these ACS HOCl (ClO^-), a potent oxidant, can be produced in vivo by neutrophils containing activated myeloperoxidase which catalyzes the reaction between physiologically present chloride ions and hydrogen peroxide (H_2O_2) [8]. In addition to neutrophils, eosinophils are also capable of generating HOCl, from H_2O_2 and Cl^-, by using eosinophil peroxidase.

Abbreviations: 2-AP, 2-aminopurine; ACS, active chlorine species; ANOVA, analysis of variance; APF, 3′-(*p*-aminophenyl) fluorescein; Cl•, chlorine atom; Cl^-, chloride anion; Cl_2, chlorine; $Cl_2^{-•}$, dichloro radical anion; ClO^-, hypochlorite ion; H_2O_2, hydrogen peroxide; HOCl, hypochlorous acid; HPF, 3′-(*p*-hydroxyphenyl) fluorescein; $^•OH$, hydroxyl radical; OC, open-circular DNA; PBS, phosphate-buffered saline; RFU, relative fluorescence units; ROS, reactive oxygen species; SC, supercoiled DNA; SDG, secoisolariciresinol diglucoside; SEM, standard error of the mean.

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However, Cl^- is not a preferred substrate in vivo [9,10]. At physiological pH, a mixture of both HOCl and ClO^- exists. HOCl kills microorganisms by oxidative damage. However, excessive production of HOCl is also known to cause inflammation and tissue damage. Hypochlorite modifies adenine nucleotides resulting in the formation of chloramines, which are implicated in neutrophil-mediated toxicity [11–13].

HOCl and its conjugated base ClO^- oxidize amino acids, peptides, proteins and lipids [14–17], and chlorinate bases in cellular DNA and RNA [14,18,19]. The reaction of HOCl/ClO^- results in the modification of both purine and pyrimidine nucleotides at the endocyclic –NH groups of guanine and thymine as well as the exocyclic NH_2 groups of guanine, adenine and cytosine derivatives [20,21] resulting in the formation of chloramines such as RNHCl and RR'NCl. The primary modified bases include 5-chlorocytosine, 8-chloroadenine and 8-chloroguanine in DNA and RNA [19,22].

It is well known that γ-radiation is capable of ionizing atoms and molecules. In biological systems or in solution, ionizing radiation generates hydroxyl radicals (•OH) [23–25], which are believed to be the source of ionizing radiation-induced damage to cellular components, including lipids, proteins and DNA [26,27]. However, these highly unstable hydroxyl radicals can be scavenged by Cl^- ions which are present at very high concentrations in physiological medium. This leads to generation of ACS [28–31], among which relatively stable ClO^- was suggested as the radiation-derived toxicant [28]. The pivotal role of ACS in hydroxyl radical-mediated chemical processes such as electrolysis [1,32–37] and Fenton reaction [38], is well known. However, the novel role of ACS, especially Cl•, $Cl_2^{\bullet -}$ and Cl_2, in γ-radiation-induced damage has only been suggested in one publication [30]. The current manuscript is to shed light on the production of ACS in physiological solutions following γ-radiation and its role in radiation-induced DNA damage. In chloride-containing solutions, ACS are formed as products of radiolysis and can impair physiological functions [31]. Therefore, we propose that radiation–induced DNA damage is mediated, in part, by radiation-generated ACS.

We have recently chemically synthesized two diastereomers of secoisolariciresinol diglucoside (SDG) [39], shown to be equipotent in their antioxidant, free radical scavenging and DNA protective properties [39,40]. The present study evaluates SDG in DNA radioprotection from γ-radiation-induced generation of ACS in physiological saline solutions using novel and specific probes. Hypochlorite-specific 3′-(*p*-aminophenyl) fluorescein (APF) and hydroxyl radical-sensitive 3′-(*p*-hydroxyphenyl) fluorescein (HPF) provide greater specificity and reproducibility for determining reactive oxygen (ROS) and chlorine species by fluorescence [41]. Dopamine, a simplified structural analog of SDG, was used to distinguish radical chlorination from hypochlorite-mediated oxidative chlorination [42] by proton magnetic resonance (^{1}H NMR) and taurine was used to detect hypochlorite-mediated *N*-chlorination.

2. Materials and methods

2.1. Chemicals

ROS indicator probes APF and HPF, plasmid DNA (pBR322), ethidium bromide, ultrapure 10X TAE buffer and 1 kb plus DNA ladder were purchased from Invitrogen (Life Technologies, Carlsbad, CA). Sodium hypochlorite, silibinin, quercetin, L-methionine, agarose (ultrapure) and calf thymus DNA were purchased from Sigma-Aldrich (St. Louis, MO). Hypochlorite concentration was documented spectrophotometrically in 10 mM NaOH at pH 12 using molar coefficient at 292 nm ($\varepsilon_{292} = 350\ M^{-1}\ cm^{-1}$). Dulbecco's phosphate buffered saline (DPBS × 1, 21-031-CV) without calcium and magnesium was purchased from Mediatech Inc. (Manassas, VA). Commercially available SDG (com) was purchased from Chromadex (Irvine, CA) and synthetic SDGs (SDG (*S*,*S*) and SDG (*R*,*R*)) were synthesized by our group [39].

2.2. Determination of hypochlorite and the scavenging effect of SDG

The fluorescence intensity of ROS probes APF and HPF (10 μM) in PBS, pH 7.4 (DPBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 2 mM KH_2PO_4) was measured at excitation/emission wavelengths of 490 nm/515 nm in a Molecular Dynamics M5 fluorescence reader. Data are expressed as relative fluorescence units (RFU).

2.3. γ-Radiation-induced generation of hypochlorite and the scavenging effect of SDG

PBS, pH 7.4 (DPBS) with APF or HPF was exposed to doses of γ-radiation ranging from 2.5 to 50 Gy using a Mark 1 cesium (137Cs) irradiator (J.L. Shepherd, San Fernando, CA) at a dose rate of 1.7 Gy/min in room air (21% O_2). In addition, experiments were also performed with various concentrations of SDG. Following radiation, the fluorescence of APF and HPF was determined. Data are expressed as relative fluorescence units (RFU).

2.4. γ-Radiation-induced generation of hypochlorite by determining taurine chloramine

Chlorination of taurine was determined using TMB assay [43]. Samples containing taurine (5.0 mM) in PBS (1×, 5× and 10×) were exposed to γ-radiation (50, 100 and 200 Gy) at 0–4 °C. After 60 min on ice, TMB reagent was added and the absorbance read in a Bio-Rad Microplate reader using 655 nm filter. A standard curve was generated using taurine chlorination in the presence of sodium hypochlorite ranging 0–40 μM. Samples were evaluated in quadruplicate and the data are expressed as taurine chloramine (absorbance) as well as ClO^- concentration (μM).

2.5. Hypochlorite-induced damage to calf thymus DNA and the effect of SDG

Calf thymus DNA (500 ng) was incubated with hypochlorite (0.1 to 0.6 mM) for 2 h at 37 °C and the reaction terminated by adding 10 mM L-methionine. The reaction was performed using 0.5 mM ClO^- and in the presence 0.5 μM SDGs, quercetin and silibinin. The selection of silibinin as a reference compound for comparing the hypochlorite scavenging ability of SDG, was not based on its structural characteristics, but based instead on its known hypochlorite scavenging action. [22]. DNA samples were subjected to agarose (1%) gel electrophoresis and analyzed as previously described [40]. The density of the high molecular weight (>6000 bps) and the low molecular weight (<6000 bps) fragments of calf thymus DNA are expressed as the percent of the total density (high molecular weight + low molecular weight DNA fragments). Densitometric analysis of high molecular weight (>6000 bps) and low molecular weight (<6000 bps) fragments of calf thymus DNA was determined by quantifying the total density of respective bands using Gel-Pro Analyzer (version 6.0; MediaCybernetics, Silver Spring, MD).

2.6. Determination of hypochlorite-induced plasmid DNA relaxation

Plasmid DNA (500 ng) in PBS (pH 7.4) was incubated with ClO^- (4.5 mM) and commercially-available SDG (25 μM) at 37 °C and the reaction terminated with 10 mM L-methionine. Samples were electrophoresed on an agarose (1%) gel and analyzed as described [40]. The density of the open-circular (OC) and the supercoiled (SC) plasmid DNA bands are expressed as the percent of the total density (open-circular + supercoiled). Densitometric analysis was performed using Gel-Pro Analyzer (version 6.0; MediaCybernetics, Silver Spring, MD).