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Oxidative stress-induced Akt downregulation mediates green tea toxicity towards prostate cancer cells

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ABSTRACT

Green tea consumption has been shown to possess cancer chemopreventive activity. Polyphenol E (PE) is a widely used standardized green tea extract formulation. This study was designed to investigate the impact of PE on prostate cancer cells (PC3), analyze the potential signals involved and elucidate whether anti- or pro-oxidant effects may be implicated. Treatment of PC3 cells with 30 and 100 μg/ml PE significantly decreased cell viability and proliferation. At the tested concentrations, PE did not exert any antioxidant activity, eliciting instead a pro-oxidant effect at concentrations 30 and 100 μg/ml, which was consistent with the observed PE cytotoxicity. PE-induced cell death was associated with mitochondrial dysfunction and downregulation of Akt activation, thus suggesting their implication in the PE-elicited cell dysfunction. Cell exposure to the ROS scavenger N-Acetyl Cysteine prevented PE-induced ROS increase, pAkt impairment, and cell death, clearly indicating the causative role of ROS in the observed phenomena. Failure of PE to induce PC3 damage in cells overexpressing Akt further confirms its implication in the PE-elicited cell death. Our findings showed an association between the antiproliferative and the pro-oxidant effect elicited by PE on PC3 cells and delineates a molecular signaling pattern potentially implicated in the toxicity of PE towards prostate cancer cells.

1. Introduction

Green tea extract from *Camellia sinensis* is a worldwide popular beverage characterized by the presence of large amounts of flavan-3-ols, known as catechins (Azam et al., 2004). Green tea catechins mainly consist of (−)-gallocatechin (GC), (+) catechin (C), (−)-epicatechin (EC), (−)-epigallocatechin (EGC), (−)-epicatechingallate (ECG), (−)-epigallocatechingallate (EGCG), and (−)-gallocatechin-3-gallate (GCG), of which EGCG is the most abundant by weight and accounts for 50–75% of the total amount of catechins (Kanwar et al., 2012). Dietary intake of green tea has been associated with a reduced incidence of risk factors for several chronic and degenerative diseases including cancer (Higdon and Frei, 2003; Jian et al., 2004; Lee et al., 2017; Yang et al., 2002), and its employment as a cancer chemopreventive agent has been proposed long ago (Mukhtar et al., 1994). Accordingly, it has been reported that green tea catechins are able to decrease cell viability and promote cell death in many cancer cell lines (Johnson et al., 2010). Polyphenon E (PE) is a standardized Green tea extract containing 50% EGCG and 30% other catechins. The highly reproducible formulation and the ease of preparation have made PE a very attractive derivative of green tea to use in clinical chemoprevention trials (Bettuzzi et al., 2006; Brausi et al., 2008; Kumar et al., 2015; Song et al., 2009).

Prostate cancer (PCa) is one of the most frequently diagnosed male neoplasia in Western countries and continues to represent one of the leading causes of cancer-related mortality despite medical advances. The reasons for this high incidence are unknown, but there is a significant difference in PCa incidence and mortality among ethnic groups, with African-American men being at the greatest risk for diagnosis, followed by Caucasian and Hispanic men. Asian-Americans appear to be at the lowest risk for PCa (Moyad and Carroll, 2004; Van Poppel and Tombal, 2011). Asians seem to have the most moderate risk

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of developing PCa, which may be due to consuming specific dietary constituents (e.g. green tea) daily for many years. Although clinical trials have yet to provide definitive results, (Bettuzzi et al., 2006; Kumar et al., 2015) many epidemiological studies, both cohort and case-control, have suggested that green tea consumption significantly correlates with a lower risk of developing PCa, suggesting a protective effect of this beverage against this disease (Jian et al., 2004; Lee et al., 2017).

Although several mechanistic models have been proposed to explain the anticancer potential of green tea (Davalli et al., 2012; Khan and Mukhtar, 2013; Shirakami et al., 2012), the fine molecular mechanisms, as well as the impacted molecular targets, are still to be identified. Specifically, whether the anti- or -pro-oxidant potential of green tea is involved in the reported antiproliferative activity on PCa cells remains to be elucidated. By using a prostate cancer cell line (PC3), the purpose of this study was to analyze whether the anti- or pro-oxidant effects of PE are involved in its anti-proliferative activity on prostate cancer cells and explore potential intracellular signals modulated by the redox activity of PE.

2. Materials and methods

2.1. Reagents

Polyphenon E is a standardized green tea extract composed of (−)-EGCG, 65%; (−)-EGC, 4%; (−)-epicatechin, 9%; (−)-epicatechin-3-gallate, 6%; (−)-gallocatechin-3-gallate, 4%; (−)-catechin-3-gallate, 0.2%; gallocatechin, 0.2%; catechins, 1.1% and caffeine, 0.7%. Polyphenon E (PE), a pharmaceutical preparation of tea catechins (http://www.polyphenon.jp/en/index.html), was supplied by Polyphenon Pharma (New York) (was kindly provided by Prof. Saverio Bettuzzi, University of Parma, Italy). Fresh 5 mg/ml PE stock solution was prepared in deionized sterile water and diluted immediately in complete medium at the final concentration required for each experiment. Insulin and N-Acetyl Cysteine (NAC) were supplied by Sigma while the Akt inhibitor, LY-294002 (LY) was from Calbiochem.

2.2. Cell culture and treatments

Human prostate cancer (PC3) cells from the American Type Culture Collection (ATCC) were routinely cultured in Fk12 nutrient mixture 1X (Invitrogen), supplemented with 7% foetal bovine serum (FBS), containing penicillin G (100 U/ml), streptomycin (100 µg/ml) and 0,25 µg/ml amphotericin B (Invitrogen). The cells were maintained at 37 °C and 5% CO_2 in a humid environment and used within passage three. PE was dissolved in Hank's Balanced Salt Solution (HBSS) for experiments using fluorescence, while in the other experiments PE was solubilized in cell culture medium. With the intent to minimize the interaction between polyphenols and proteins (Jannin et al., 2004; Zhang et al., 2016; Zinellu et al., 2014) 2.5% FBS was used during all the experiments. In agreement with previous works (Brizuela et al., 2010; Thakur et al., 2012), cells (70–80% confluent) were treated with PE (10, 30, and 100 µg/ml) for different time points depending on the experiment performed, while cells used as controls were incubated with the vehicle only. In selected analysis, cells were pre-incubated for 30 min with the Akt activator insulin (30 µM) and specificity of the insulin-mediated Akt activation was demonstrated by using the selective PI3K inhibitors LY-294002 (10 µM) (Hermann et al., 2000). To study the involvement of oxidative stress on the elicited PE cell damage, we employed the ROS scavenger NAC (5 mM) (Pasciu et al., 2010; Posadino et al., 2015).

2.3. Determination of cell viability

Cell viability was assessed by using the CellTiter-Glo®Luminescent Kit (Promega), which is a homogeneous method to determine the number of viable cells in based on quantitation of the produced ATP, an unequivocal marker of metabolically active cells. The homogeneous "add-mix-measure" format results in cell lysis and generation of a luminescent signal proportional to the amount of ATP present. The amount of ATP is directly proportional to the number of cells. Briefly, after treatment of 24 h, plates were removed from the incubator and allowed to equilibrate at room temperature for 30 min, and an equal volume of CellTiter-Glo reagent was added directly to the wells. These contents were mixed for 2 min on an orbital shaker to induce cell lysis. Plates were incubated at room temperature for 10 min to stabilize luminescent signal. The luminescence was measured on GENios Plus microplate reader (Tecan) and results expressed as a means ± SD of the percent of control (Hata et al., 2016; Lin et al., 2008; Posadino et al., 2012). In selected experiments, the number of cells was determinate by using an automatic cell counter (LUNA™ Automated Cell Counter by Logos Biosystems), and results were expressed as a means ± SD of the number of cells per ml (n = 4).

2.4. Determination of cellular metabolic activity

Cell metabolic activity was assessed in 96-well plates (BD Falcon) by using the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Promega) (Posadino et al., 2013; Posadino et al., 2015). After treatment of 24 h, cells were added with 20 µl MTT solution (5 mg/ml) in medium M199 and incubated at 37 °C in a cell incubator for 60 min. At the end of the incubation period, the medium was removed, and the cell monolayer was washed twice with HBSS. The converted dye was solubilized with acidic isopropanol (0.04 N HCl in absolute isopropanol), and plates were analyzed at 570 nm using a GENios Plus microplate reader (Tecan) with background subtraction at 650 nm. Results were expressed as a percent of untreated control cells (means ± SD for n = 5).

2.5. Determination of cellular DNA synthesis

The rate of cellular DNA synthesis was assessed by using a chemiluminescent immunoassay method, which is based on the measurement of BrdU incorporation during DNA synthesis. When cells are pulsed with BrdU, it is incorporated into newly synthesized DNA strands of actively proliferating cells. The incorporation of BrdU into cellular DNA can be detected using anti-BrdU antibodies, allowing assessment of the population of cells synthesizing DNA. Confluent cells were treated with different concentrations of PE and the proliferation was evaluated at different time points, 24 h and 48 h. BrdU is added to cells cultured in microplates, followed by incubation for 10 h. After the culture supernatant is removed, the cells are fixed by Fix-Denat solution for 30 min. Fix-Denat was discarded and cells were incubated with an anti-BrdU antibody conjugated to peroxidase (anti-BrdU-POD) for 90 min. After rinsing three times with washing buffer, substrate solution was added and allowed to react for 3–10 min at room temperature. Finally, light emission was read by using a GENios Plus microplate reader (Tecan). Results were normalized for protein content and expressed as a means ± SD of the relative fluorescence units (RFU) values (Floris et al., 2015).

2.6. Measurements of intracellular ROS

Intracellular ROS levels were determined by using the ROS molecular probe 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) (Molecular Probes) as previously described with minor modification (Boin et al., 2014; Giordo et al., 2013). Within the cell, esterases cleave the acetate groups on H_2DCF-DA, thus trapping the reduced form of the probe (H_2DCF). Intracellular ROS oxidize H_2DCF, yielding the fluorescent product, DCF. For ROS measurements, cultured cells were pre-incubated for 30 min with PBS plus containing 1 µM H_2DCFDA, then washed with PBS and treated as described. Fluorescence was measured by using a Tecan GENios Plus microplate reader (Tecan,) in a light-